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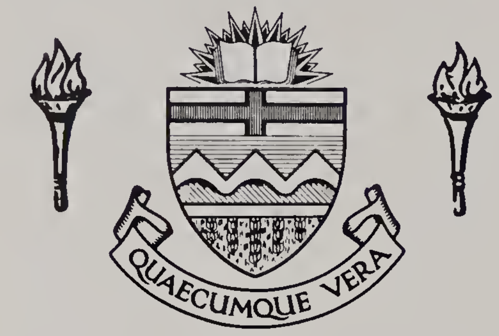

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# STUDIES OF THE F-SPECIFIC BACTERIAL CONJUGATION IN *ESCHERICHIA COLI* K12

by

© LAURIE ANN SCHULTZ

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled STUDIES OF THE F-SPECIFIC BACTERIAL CONJUGATION PROCESS IN *ESCHERICHIA COLI* K12 submitted by Laurie A. Schultz in partial fulfilment of the requirements for the degree of Master of Science.

# ABSTRACT

F-specific protein transfer during conjugation of *E. coli* bacterial cells was used to study the role of F pili in bacterial mating. Radioactively labelled donor cells were mated with non-labelled recipients; these were then separated and the recipient cells were analyzed by gel electrophoretic techniques to locate any radioactive donor proteins which may have been transferred to the recipient cells during the mating. Transfer of three donor proteins was observed to occur during conjugation. F pilin was not one of these proteins, but this does not necessarily mean that F pilin is not transferred, since it is possible that the pilin did not penetrate the gels. Two or more of the protein spots could be cleavage products of a postulated pilot protein which may serve to guide the DNA from donor to recipient. This would favour the carrier model of the role of F pili in conjugation. On the other hand, the isoelectric point and molecular weight of one of the detected protein spots match that of the outer membrane matrix protein, which could indicate that the retraction model is correct. These results suggest that Brinton's conduction (1965) and conveyor belt (1971) models are unlikely mechanisms for the role of F pili in conjugation since they predict that only DNA is transferred. However, a distinction could not be made at this time between the retraction model of Curtiss (1969) and Marvin and Hohn (1969), and the carrier model suggested by Paranchych, both of which predict that protein transfer does occur.

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# LIST OF ABBREVIATIONS

F - F transfer plasmid

tra - transfer deficient

fin - fertility inhibition

omp - outer membrane protein

pif - phage infection inhibition

frp - F replication

inc - incompatibility

ori V - origin of negative replication

ori T - origin of transfer

Con$^-$ - conjugation deficient

Hfr - high frequency of recombination

DNA - deoxyribonucleic acid

RNA - ribonucleic acid

DNase - deoxyribonuclease

RNase - ribonuclease

MOPS - potassium morpholinopropane, sulfonate

Tris - Tris (hydroxymethyl) amino methane

TSB - trypticase soy broth

SDS - sodium dodecyl sulfate

TEMED - N,N,N',N'-tetramethylethylenediamine

DMSO - dimethyl sulfoxide

PPO - 2.5 diphenyloxazole

TCA - trichloroacetic acid

EtOH - ethanol
IEF - isoelectric focusing
Ci - Curie; $2.22\text{x}10^{12}$ disintegrations per minute
cpm - radioactive counts per minute
rpm - revolutions per minute
g - centrifugal force relative to gravity
$A_{280}$ - light absorbance of a solution in a 1 cm light path at 280 nm
MW - molecular weight
pI - isoelectric point
V - volts
mamp - milliamperes
w/v - weight per volume
v/v - volume per volume

All temperatures are in degrees centrigrade

# CHAPTER 1

# INTRODUCTION

The phenomenon of bacterial conjugation, the process by which genes are transferred between bacteria, was first discovered in 1946 by Lederberg and Tatum (1946). The genes that encode the proteins for conjugation are located on a plasmid. These plasmids are called sex factors. They mediate transfer of themselves, of DNA from co-resident, non-sex factors and also, under some circumstances, of chromosomal DNA from one bacterium to another.

Conjugative plasmids are supercoiled, covalently closed DNA molecules which are usually between 40 and 70 megadaltons in size. There are normally 1 to 3 copies of the plasmid per chromosome (Grindley *et al*. 1973).

The *E*. *coli* sex factor F was the first conjugative plasmid to be discovered (Hayes, 1953), and as such, has been the most well studied plasmid to date. More recently, plasmids which confer antibiotic resistance (R factors) and colicinogeny (Col factors) have also been shown to be sex factors (Watanabe, 1963; Meynell *et* *al*. 1968). Many genera of bacteria have been found to contain sex factors and many of these can be transferred intergenerically.

Originally it was thought that the mechanism of Hfr x $F^-$ matings was the mechanism of conjugation for all plasmids. However, this no longer seems to be the case, since there are several types of transfer systems which have been identified. The four main systems which have been identified to date are the F, I, N, and P systems. The pili specified by each type of plasmid, and thus the male-specific phages which

adsorb to the pili are different in the four groups. Also, there exists no DNA homology between the plasmids carrying these transfer systems. Therefore it seems unlikely that their conjugation mechanisms would be the same.

The F transfer system is the best understood transfer system, both in genetic and biochemical terms. The information of F factor genetics and the mating process will be discussed below.

## A. Genetics of the F Factor

The F factor is 62 megadaltons, or 94.5 kilobases (kb) in size. All the genes on the F factor which have currently been mapped are clustered according to their function. The *pif* cistrons, which lead to inhibition of phage infection are located between 33 and 43 kb (Anthony *et al*. 1974; Skurray *et al*. 1976a). Mapping between 40.3 and 49.3 kb are the genes required for F factor DNA replication (*frp*) and for incompatibility (*inc*) (Guyer *et al*. 1976; Timmis *et al*. 1975; Lovett and Helinski, 1976; Skurray *et al*. 1976b). The origin of vegetative replication, *ori* V, (Clark *et al*. 1976) has been located at 42.6 kb (Helinski, as quoted by Achtman and Skurray, 1977). Santos (Santos *et al*. 1975) has located the *inc* genes between 46.5 and 48.5 kb. These genes vary in their location among other members of the FII and FIII group of plasmids.

The transfer or *tra* region, which maps between 62 and 93 kb, is a very highly conserved region among F and F-like plasmids (Sharp *et al*. 1973). This is the region which is responsible for the conjugative ability of the plasmids. The current map of the F factor is shown in figure 1 (Achtman *et al*. 1977; Achtman *et al*. 1978a; Miki *et al*. 1978).

There are 19 known *tra* genes, 17 of which compose the transfer or

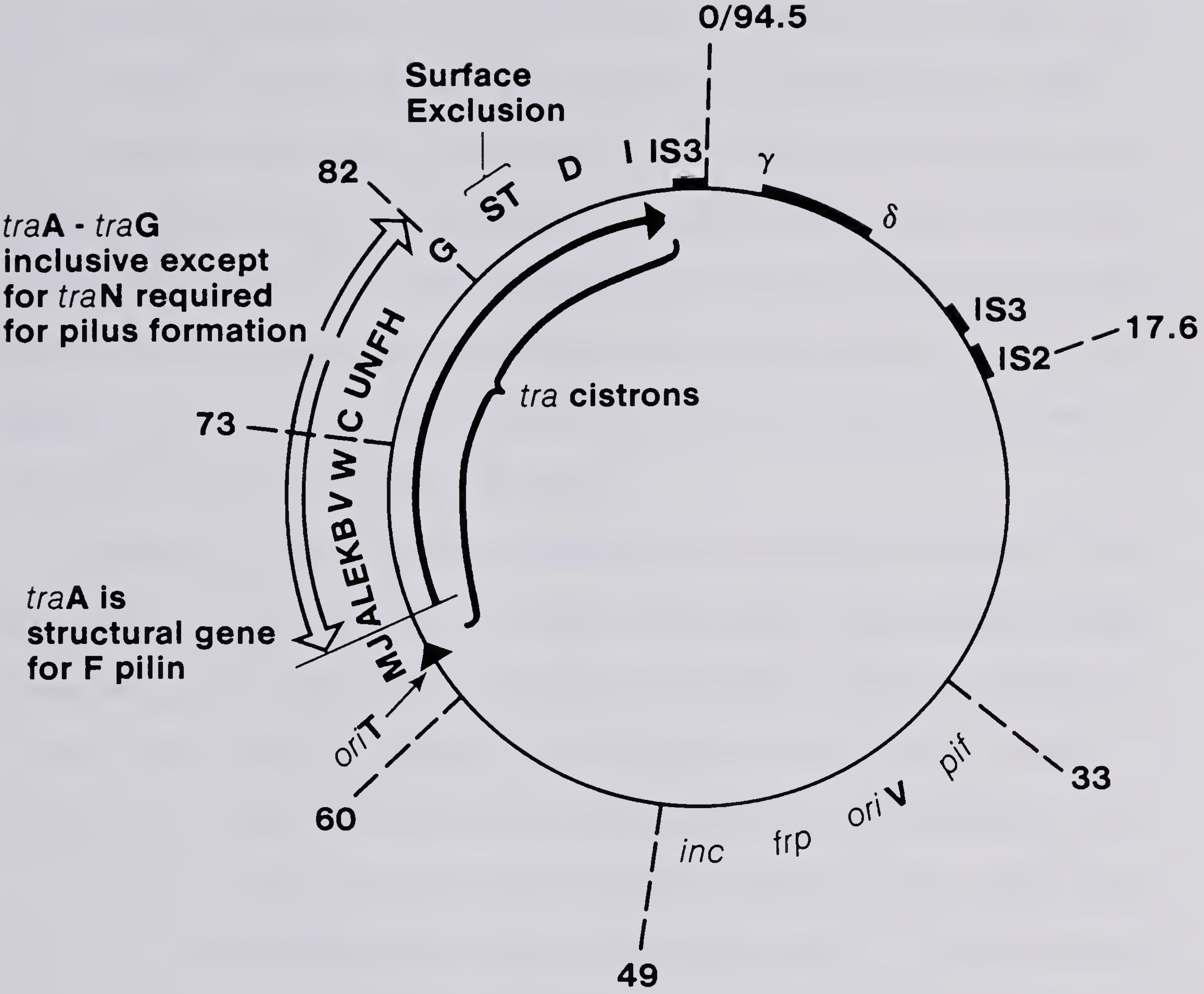


Figure 1. The map of the F factor.

The transfer operon (A to I) is indicated by the heavy arrow drawn clockwise inside the circular map. The numbers indicate length in kilobases. IS2, IS3 and γ - δ are insertion sequences.

*tra* operon (Achtman and Helmuth, 1975; Helmuth and Achtman, 1975). Of the first 13 genes of the operon, 12 are required for synthesis of F pili: *tra*A, *tra*L, *tra*E, *tra*K, *tra*B, *tra*V, *tra*W, *tra*C, *tra*U, *tra*F, *tra*H and part of *tra*G (Achtman *et* *al*. 1971, 1972; Miki *et* *al*. 1978). *Tra*N maps between *tra*U and *tra*F, but is not required for F pili formation. These 13 genes map between 65 and 82 kb (Sharp *et* *al*. 1972). The *tra*A gene codes for F pilin, the structural subunit of the pilus (Minkley *et* *al*. 1976). The function of the 11 other genes is unknown at the present time. It has been suggested that they code for enzymes which process the *tra*A gene product to its final subunit form as pilin. Mutants in any of these genes do not produce the pilus and so are Tra$^-$ (incapable of promoting DNA transfer).

Isolation of Tra$^-$ mutants resulted in the identification of four other genes of the *tra* operon, these being *tra*G, *tra*D, *tra*I and *tra*M (Achtman *et* *al*. 1971, 1972; Willetts and Achtman, 1972). *Tra*G gene product seems to be bifunctional, as there are two classes of *tra*G mutants which have been isolated, those which do, and those which do not produce F pili. Although *tra*D mutants produce F pili, RNA pilus-specific bacteriophages will not infect *tra*D mutants. Neither the adsorption or ejection stages of R17 phage infection are affected by mutations in *tra*D and so it has been postulated (Paranchych, 1975) that the *tra*D product may be required for penetration of RNA through the cell membrane and, by analogy, of the DNA as it passes out of the cell during conjugation. Pilus structure is not known to be affected by either *tra*I or *tra*M mutations. It is believed that *tra*I is involved in DNA metabolism during conjugation and may be an endonuclease which recognizes the origin of transfer (Willetts, 1972a; Reeves and Willetts,

1974). The three genes, *tra*G, *tra*D and *tra*I all map within the operon, between 80 and 93 kb (Sharp *et al*. 1972). *Tra*M maps outside the operon, between 60 and 68 kb (Achtman, as stated in Achtman and Skurray, 1977).

The *tra*S and *tra*T genes are not involved in donor ability, and as such, cells with mutations in these genes are quite capable of transferring DNA. These genes are involved in surface exclusion, whereby cells which contain conjugative plasmids are themselves poor recipients in conjugation. These genes map between *tra*G and *tra*D (Willetts, 1974). Achtman *et al*. (1977) have determined that *tra*S and *tra*T act independently of each other to cause surface exclusion in two ways. The *tra*T gene product inhibits the formation of stable mating aggregates, while the *tra*S gene product inhibits DNA transfer.

These eighteen genes, with the exception of *tra*M, form the *tra* operon. This operon is transcribed in a direction from *tra*A through to *tra*I. The operon is controlled by a positive control gene, *tra*J, which maps outside the operon, very close to *tra*A. All functions expressed by the *tra* operon, that is, presence of pili, transfer of DNA, and surface exclusion, are absent in cells which have a mutation in *tra*J (Achtman *et al*. 1972). Willetts (1977) has recently shown that *tra*J is necessary for transcription of the operon.

The *E*. *coli* F factor is constitutive for the *tra* operon, that is, it is always expressing the genes encoded by this region of DNA. However, many F-like sex factors isolated from nature are normally repressed for these functions (Meynell *et al*. 1968). This repression is mediated by the products of two genes, *fin*O and *fin*P, both of which are necessary for the self-repression (Willetts, 1972b; Gasson and Willetts, 1975). The *fin*O product is non-specific and can act with the *fin*P

product of any F-like sex factor. Most F-like sex factors produce both the *fin*O and *fin*P gene products and so are self-repressed. The F factor, however, produces an inactive *fin*O product and so is naturally derepressed. If a plasmid with an active *fin*O product is introduced into a cell carrying the F factor, the active *fin*O product may combine with the *fin*P product of the F factor and cause repression of its *tra* region (Finnegan and Willetts, 1971). The *fin*O-*fin*P repressor complex acts at a site designated *tra*O to prevent synthesis of the *tra*J protein (Finnegan and Willetts, 1973; Achtman, 1973). This in turn results in the lack of transcription of the *tra* operon (Davis and Vapnek, 1976; Willetts, 1977).

## B. Protein Components Required for Mating

The initial step in the conjugation process is the formation of a specific contact between the tip of the F pilus of the $F^+$ cell and a suitable recipient cell surface. It has been suggested that a major outer membrane protein, the product of the *omp*A gene, is the receptor protein in the recipient cell (Manning and Reeves, 1976; Manning *et al*. 1976).

F pili are a necessary part of the conjugation machinery, although their full role in this phenomenon remains unknown. F pili are filamentous protein organelles with a cross-sectional diameter of 90 Å (Brinton, 1965; Lawn, 1966) and are variable in length with an average of 1 to 2μm (Brinton, 1965; Novotny *et al*. 1969; Paranchych *et al*. 1971). The pilin subunit has a molecular weight of about 11,800 daltons (Brinton, 1971; Beard & Connelly, 1975), as determined by SDS polyacrylamide gel electrophoresis and contains two phosphate and one glucose per molecule. However, on the basis of amino acid composition, the

molecular weight appears to be 13,000 (Brinton, 1971; Date et al. 1977).

As well as functioning in conjugation, pili serve as the receptor for male-specific bacteriophages, with RNA phages adsorbing along the sides and DNA phages at the tip of the pilus. There is evidence for RNA phages that the A protein and attached RNA leave the phage and are transported to the cell surface via the F pilus (Krahn et al. 1972; Paranchych, 1975; Wong and Paranchych, 1976). Possible models for the role of the F pilus in conjugation will be discussed later.

There are several chemical treatments which cause the disappearance of pili from the cell surface. Incubation at 25° (Novotny and Lavin, 1971; Walmsley, 1976), at 50° (Novotny and Fives-Taylor, 1978) or in 5 mM cyanide (Novotny and Fives-Taylor, 1974, Achtman, 1975) causes an apparent retraction of pili. Pili are dissolved in 0.01% sodium dodecyl sulfate (Tomoeda et al. 1975; Achtman, 1975). Treatment with 1 mM $Zn^{2+}$ (Ou and Anderson, 1972; Achtman, 1975) cause shedding of full length pili into the medium . Pili can also be removed from the cell surface by blending (Brinton, 1965). All of these treatments result in the loss of conjugative function. Brinton (1965) also showed that following blending the ability of the cells to conjugate returned with the same kinetics as the reappearance of pili. The addition of male-specific bacteriophages to $F^+$ cells also prevents conjugational DNA transfer (Knolle, 1967; Novotny et al. 1968; Ippen and Valentine, 1967). These studies all indicate that pili are a necessary component in the conjugational process.

A component of the recipient cell's outer membrane is also required for conjugation. Mutants in a gene called ompA (Datta et al. 1976), which maps at 21.5 minutes on the E. coli chromosome (Foulds, 1974; Henning et al. 1976; Manning et al. 1976) have been found to be

poor recipients in conjugation (Skurray _et al_. 1974; Manning _et al_. 1976). The protein pOmpA has a molecular weight of 28,000 daltons, and is largely insoluble in water (Schnaitman, 1974; Reithmeier and Bragg, 1974; Hindennach and Henning, 1975). Mutations in the _ompA_ gene have pleiotropic effects. Mutants in this gene are resistant to phages K3 and TuII*, tolerant to colicins L and K, sensitive to ethylene diamine tetraacetic acid, phenethyl alcohol, eosin yellow and novobiocin, and they do not form stable mating aggregates (Manning and Reeves, 1976; Manning _et al_. 1976). Skurray _et al_. (1974) suggested that the protein pOmpA is the receptor for the F pilus. However, purified F pili bind to Con$^-$ mutants, which lack pOmpA. Cells which are poor recipients in conjugation are termed Con$^-$. Thus it seems there is a stage in conjugation at which F pilus binding to the recipient cell is involved, but at which pOmpA is not. Achtman and Skurray (1977) suggest that after the initial F pilus - recipient cell contact, an unidentified donor component which may be coded for by the sex factor, must bind to the recipient cell's pOmpA for stabilization of the mating aggregates to be successful.

## C. Models for the Role of F Pili in Conjugation

Once the pilus has formed a specific contact between the donor and recipient cells, its further role in conjugation is still uncertain. There are, at present, two models for the role of F pili in conjugation. The first, proposed by Brinton (1965, 1971), is based on the cells remaining apart during the mating process, with the F pilus connecting them. The retraction model, proposed by Marvin and Hohn (1969) and Curtiss (1969) requires that the cells be brought into wall to wall

contact before DNA transfer can occur.

In 1965, Brinton proposed a tubular structure for F pili, with an axial hole down the center. It was through this hole that DNA was presumably transferred during conjugation. More recently, Brinton (1971) proposed that F pili consist of two parallel protein rods. The evidence for this comes from the fact that he dissociated pili into filaments which were one-third the diameter of the intact pilus. Also, he identified a dark line down the center of each F pilus by electron microscopy, where the negative stain had penetrated. Using this basis for pili structure, he proposed three methods by which the nucleic acid could be transported along the pilus: 1) by conduction, in which the DNA or phage RNA is transferred in the groove of the filament; 2) by a carrier mechanism in which the pilus is assembled in the donor membrane and disassembled in the recipient cell membrane; or 3) by a conveyor belt mechanism in which the two filaments of the pilus move with respect to each other. In all of these methods, the nucleic acid, whether it be DNA in conjugation or the phage RNA or DNA, is assumed to travel along the length of the pilus. No one has yet been able to demonstrate or isolate pili which have DNA associated with them. Evidence for this model of the role of F pili in conjugation comes from the observation of Ou and Anderson (1970) that DNA transfer did sometimes occur between cells which did not visibly come into wall to wall contact. Mating pairs have been seen in the light microscope in which the two cells remain a fixed distance apart, presumably connected by a pilus, which cannot be detected at that level of magnification (Brinton, 1965; Ou and Anderson, 1970).

Marvin and Hohn (1969) and Curtiss (1969) independently proposed

a second mechanism for the role of F pili in conjugation. This model is based on pilus retraction. They envision that on contact with a recipient cell, or when a phage particle adsorbs to the pilus, a signal is produced which causes the pilus to retract into the donor cell, bringing the donor and recipient cells into wall to wall contact, or bringing the phage particle to the cell surface.

Evidence has been obtained by several groups that pili do retract. Jacobsen (1972) showed the average F pilus length decreased on infection of _E_. _coli_ by male-specific phages, and that the phage capsids accumulated at the cell surface. Novotny showed that reducing the temperature of the culture to 25° (Novotny and Lavin, 1971) or heating to 50° (Novotny and Fives-Taylor, 1978) caused the disappearance of pili. Treatment of cells with 5 mM cyanide (Novotny and Fives-Taylor, 1974) or treatment of glycerol-grown cultures with arsenate (O'Callaghan _et_ _al_. 1973) also caused pili to disappear from the cell surface. The pili could not be detected in the medium and so it was assumed they had retracted back into the cell membrane. This retraction could be prevented by the addition of pilus-specific antibodies, or adsorption of RNA phage particles.

Recently, Marvin (personal communication) has shown by fiber diffraction studies that pili are hollow cylinders with an 80 Å outer diameter and a 20 Å inner diameter. The pilus structure is of four coaxial helices, each with a pitch of 128 Å, with the pilin subunits elongated and overlapping along the line of the helices. This structure is very different from Brinton's two filament proposal (Brinton, 1971), based on electron microscopic data. However the two models may be reconciled if two of Marvin's four helices pull away from the other two and so

cause a space where stain could penetrate, leading to a filament hypothesis. The structure of pili as determined by the fiber diffraction studies, must be taken into account when proposing or dealing with the models of F pilus function in conjugation.

Achtman has recently proposed that extended F pili may play no role in DNA transfer, but are necessary only for the initial cell-cell interactions (Achtman *et al*. 1978b). Most conjugating cells were not connected by extended F pili. Those pili which were still extended could be depolymerized by treatment with SDS, without affecting DNA transfer. However when SDS was added at the beginning of mating, full inhibition of mating aggregation and of DNA transfer was observed. These data led them to suggest that F pili are essential for the formation of mating aggregates, but play no role in DNA transfer. Although the data presented are convincing, some of the results are based on electron microscopic observations and as such are open to some criticism since the aggregation of cells that were seen could have, in part, been due to clumping together of the cells when the stain was added to the grid. However, Coulter counter data, which are statistically more accurate, did support the electron microscopic results for which the two methods were used. Achtman suggested that if pili do retract, the retracted pili may be involved in the transfer of DNA. It is possible, however, that pili play no role except that of forming aggregates, and that membrane fusion of the donor and recipient cells is responsible for the DNA transfer.

Although the role of F pili in conjugation is still not clearly understood, it seems evident from Achtman's data (Achtman *et al*. 1978b), that the extended F pili play no role in DNA transfer, as suggested by

Brinton (1965, 1971). However, work with R17 infection of *E. coli* has demonstrated that pili possess a specific protease activity, which cleaves the phage A protein, which is attached to the phage RNA, into two smaller polypeptides (Krahn *et al*. 1972). The model of Achtman does not take this activity into account. Also, the idea of a pilot protein which leads the DNA from donor to recipient is attractive in view of the fact that several phages, such as R17, M13 and $\phi$X174 have pilot proteins to help the phage nucleic acid to penetrate the bacterial membrane. This, too, has no place in Achtman's model, although the presence of a pilot protein could be envisioned and added. The role of F pili in DNA transfer and conjugation must remain uncertain until further investigations have taken place.

## D. DNA Transfer during Conjugation

Once the donor and recipient cells have made their specific contacts, DNA transfer can begin. One strand of the donor DNA enters the recipient cell (Gross and Caro, 1966; Cohen *et al*. 1968a, b) with the 5' end first (Ohki and Tomizawa, 1968; Rupp and Ihler, 1968). The plasmid DNA is cut at *ori*T, the origin of transfer. The *ori*T region may define a nucleotide sequence which is recognized by a sex-factor coded nuclease (Willetts, 1972b). This nuclease has been postulated to be the product of the *tra*I gene (Willetts, 1971; Alfaro and Willetts, 1972; and Willetts, 1974).

In the process of nucleic acid transfer, either from phage to cell, or cell to cell, the DNA or RNA must at some point cross the cell membrane. The cytoplasmic membrane is normally a barrier to most molecules and so it must change in some way to accomodate the nucleic acid. A

pilot or leader protein has been postulated by Lacks and Greenberg (1976) in the transformation of *Diplococcus pneumoniae*, and by Kornberg (1974) for viral infection, that leads the DNA through the cell membrane to the interior. The A protein of phage R17 binds to the F pilus and guides the RNA, to which it is attached, into the cell (Krahn *et al*. 1972). DNA phages such as M13 or ϕX174 also have pilot proteins (Jazwinski *et al*. 1973, 1975) which bind the phage to the cell and then are released into the cell envelope to form a protein pore lined with DNA binding sites through which the phage DNA can penetrate. Kornberg (1974) has proposed an analogous mechanism for involvement of a pilot protein in DNA transfer during conjugation.

## E. The Mating Process

Recently, Achtman and Skurray (1977) have defined five stages within the overall mating process. The first step is the F pilus binding stage. Contact between donor and recipient cells is established by the pilus. This is followed by the wall to wall contact stage. Although there is no direct evidence that wall to wall contact occurs during bacterial mating, many mating cells have been visualized by microscopy to be in apparent wall to wall contact. Since these mating aggregates are unstable, a third stage has been proposed in which stabilization occurs. The unstable mating aggregates easily separate unless the protein pOmpA is present in the recipient cell membrane. This protein of the recipient is therefore necessary in some way for conjugation to occur. Stable mating aggregates can be formed by *tra*D, *tra*I and *tra*M mutants, but no DNA transfer occurs from these mutants. This suggests that DNA transfer is a separate stage which occurs after stabilization

of the mating aggregates. The last stage, which applies only to autonomous sex factor matings, but not Hfr x $F^-$ matings, is the disaggregation of mating aggregates. Mutants are available which are specifically blocked in each of the stages, except the wall to wall contact stage.

The study described in this thesis was undertaken to further characterize the mating process by determining if there was any protein transfer from $F^+$ to $F^-$ cells during conjugation. This could possibly indicate which of the postulated mechanisms of DNA transfer is likely to occur, or at least limit the choice between them. The retraction model, proposed by Curtiss (1969) and Marvin and Hohn (1969), and more recently, with some convincing proof by Achtman (Achtman _et al_. 1978b), would result in fusion of the donor and recipient cell membranes. Under these circumstances, several donor proteins, from the membrane or cell envelope could be transferred to the recipient as the cells break apart after DNA transfer is complete. The conduction and conveyor belt models of Brinton (1965, 1971) would not give rise to any type of protein transfer. Evidence obtained by studies with R17 infection of _E. coli_ cells has led Paranchych to propose a carrier mechanism of conjugation in which mating pair formation gives rise to the movement of F pilin subunits from donor to recipient by assembly of pilin subunits in the donor cell coupled with the depolymerization of pilin subunits in the recipient cell membrane. Included in this model is the idea that the DNA to be transferred is attached to a plasmid-specific pilot protein, which is also transferred to the recipient cell during conjugation. This model would suggest that two proteins, F pilin, and a pilot protein, should be transferred from donor to recipient during conjugation. These three models should result in different patterns

of protein transfer. This study, which involved mating radioactively labelled donor cells with non-labelled recipient cells, and looking for transferred radioactive donor material, was attempted in the hope of making a distinction between the various models that have been proposed for the role of F pili in conjugation.

# CHAPTER II

# MATERIALS AND METHODS

## A. Materials

### 1. Bacteria and Bacteriophages

#### a) Bacteriophage

The phage T6 was used throughout the studies described in this thesis.

#### b) Bacteria

The various strains of *Escherichia coli* used are:

| Strain | Genotype |
|---|---|
| ED2601 | Strain K12 $F^-$ lac his trp gal lys $Sm^R$ $Spc^R$ $T6^R$ fla |
| ED2602 | Flac/ED2601 |
| JC6256 | Strain K12 $F^-$ lac trp $Sm^S$ $Spc^S$ $T6^S$ $Su^-$ |
| JC6583 | Flac/JC6256 |

All strains of bacteria were maintained on hard agar plates for routine use. Permanent stocks were stored on hard agar slants in sealed vials. All cultures were started by transferring a single colony on an agar plate to 5 ml of liquid medium which was grown at 37° with or without shaking overnight (17 h).

### 2. Bacterial Culture Media

#### a) MOPS medium (Neidhardt *et al*. 1974).

A 10x concentrated stock of MOPS medium consisted of the following chemicals: potassium morpholinopropane sulfonate (MOPS) freshly prepared and adjusted to pH 7.4 with KOH, 0.4 M; N-Tris (hydroxymethyl) methylglycine (Tricine), freshly prepared and adjusted to pH 7.4 with

KOH, 0.04 M; $FeSO_4$, freshly prepared, $10^{-4}$ M; $NH_4Cl$, 0.095 M; $K_2SO_4$, $2.76 \times 10^{-3}$ M; $CaCl_2$, $5 \times 10^{-6}$M; $MgCl_2$, $5.28 \times 10^{-3}$M; NaCl, 0.5 M; $(NH_4)_6(MO_7)_{24}$, $3 \times 10^{-8}$ M; $H_3BO_3$, $4 \times 10^{-6}$M; $CoCl_2$, $3 \times 10^{-7}$ M; $CuSO_4$, $10^{-7}$M; $MnCl_2$, $8 \times 10^{-5}$ M; and $ZnSO_4$, $10^{-7}$ M. This solution was then filter sterilized and stored in aliquots at -20°.

To prepare MOPS medium for use, the 10x concentrated stock was diluted ten fold. To this was added $K_2HPO_4$ at a concentration of $1.32 \times 10^{-3}$ M, a carbon source and any required amino acids at a concentration of 50 μl/ml.

(i) Low sulfate MOPS medium - The $K_2SO_4$ concentration in the 10x concentrate was reduced by 10 to $2.76 \times 10^{-4}$ M.

b) M9 buffer

One liter of M9 buffer was prepared by mixing 7 g of $Na_2HPO_4$, 3 g of $KH_2PO_4$, 0.5 g of NaCl and 1 g of $NH_4Cl$ in water. This solution was dispensed in bottles and autoclaved.

c) L-broth

L-broth was prepared by autoclaving a solution of 10 g Tryptone (Difco), 5 g yeast extract (Difco) and 10 g of NaCl in one liter of water. The autoclaved solution had a pH of 7.2 - 7.3.

d) Hard Agar

An autoclaved solution of hard agar, consisting of 30 g/l Trypticase soy broth (TSB) and 15 g/l Bacto-Agar (Difco) was dispensed while warm into disposable Petri dishes.

e) Top Agar

A one liter solution of 30 g Trypticase soy broth and 11 g of Bacto-Agar (Difco) in water was autoclaved and stored in 50 ml volumes at 4° until used. For viable cell counts, the agar was melted

in a microwave oven and dispensed into sterile culture tubes in 3 ml aliquots and maintained in the liquid state until used by incubating at 60° in a Temp-Blok (Lab-Line Instruments, Inc.).

f) H-broth

H-broth was made by mixing and autoclaving 8 g/l nutrient broth, 5g/l Bacto Tryptone and 5 g/l NaCl, after which 1 g/l glucose was added.

g) T6 Bottom Agar

T6 bottom agar consisted of 10 g/l Agar (Difco), 13 g/l Bacto Tryptone, 8 g/l NaCl, 2 g/l Na citrate·2 $H_2O$ and 1.3 g/l glucose. This solution was autoclaved and dispensed while warm into disposable Petri dishes.

h) T6 Top Agar

T6 top agar consisted of 6.5 g/l Agar (Difco), 13 g/l Bacto Tryptone, 8 g/l NaCl, 2 g/l Na citrate·2 $H_2O$ and 3 g/l glucose. This solution was dispensed into 125 ml bottles, autoclaved and stored at 4° until used.

3. Bacteria and Phage Diluent

All dilutions of bacteria or phage were made with a sterile solution composed of 0.9% (w/v) NaCl, 5 mM $MgCl_2$ and 5 mg % bovine serum albumin. Diluent was dispensed in 9 and 10 ml volumes into sterile 20 mM dia,dilution tubes and stored at 4°. These solutions were warmed to room temperature before use.

4. Chemicals and Reagents

All reagents were prepared from analytical grade chemicals in double-distilled water unless otherwise specified. Ampholytes were obtained from LKB Produktur, Bromma, Sweden. Sodium dodecyl sulfate (SDS),

technical grade, was obtained from Matheson, Coleman and Bell.

N,N,N',N' - tetramethylethylenediamine (TEMED), N,N'-bis methylene (bis) acrylamide, and riboflavin were obtained from Eastman Kodak. The bis acrylamide was recrystallized before using according to the procedure published by Loening (1967). Acrylamide was purchased from Bio Rad Laboratories.

NCS reagent employed for solubilizing polyacrylamide gel slices was from Nuclear Chicago.

The X-ray film used was Kodak X-Omat R, XR-1 film, purchased from Kodak.

### 5. Radioactive Materials

Radioactive precursors for the preparation of labelled cell proteins were obtained from the following sources: $^{3}H$-L amino acid mixture (1.0 mCi/ml in 0.1 M HCl), $^{14}C$-L-amino acid mixture (0.1 mCi/ml in 0.1 M HCl), and $^{35}S$ as $Na_2{}^{35}SO_4$ in water (500 - 900 mCi/mM) from New England Nuclear; [$^{35}S$]-methionine (770 Ci/mmole) from Amersham Corporation.

## B. Growth of Bacteria

Experimental cultures were grown at 37° in a rotary-shaking water bath from a 1:50 dilution of an overnight culture in the same medium. Maximum aeration and minimum pilus breakage was achieved by using shallow cultures, generally 20% of the flask volume, shaken at 140 rpm in baffled culture flasks (Bellco Glass Co.). Under these conditions, cultures generally reached a cell density of $5 \times 10^{8}$ cells/ml about 5 - 6 hours after innoculation using minimal media and 2 - 2.5 hours in rich media.

The density of E. coli cells in various media was determined from a standard curve constructed by plotting the absorbance at 650 nm of a 1.0 ml volume of culture in a cuvette (with a light path of 1 cm) vs. the viable cell count. Viable cell counts were determined by plating 1.0 ml of an appropriate dilution of the culture mixed with 3.0 ml of soft top agar, which was spread on hard agar plates. The plates were incubated at 37° overnight and scored for bacterial colonies.

### C. Radioactive Labelling of Bacterial Cultures

All cultures to be labelled were grown to a density of $1 \times 10^8$ cells/ml before the labelled material was added. MOPS and low sulfate MOPS media were used for $^3H$ and $^{35}S$ labelling respectively, while M9 medium supplemented with glucose, $Mg^{2+}$, thiamine and any required amino acids was used for $^{14}C$ labelling. The amounts of label added varied between experiments, but was normally from 20-60 µCi/ml for $^3H$, 50-100 µCi/ml for $^{35}S$, and 1-4 µCi/ml for $^{14}C$. Approximately 100% of the label was incorporated when $^3H$-amino acids were added, and 30-50% of the $Na_2{}^{35}SO_4$ was incorporated. After the label was added, the cells were allowed to grow to allow incorporation of the label into protein. For $^3H$, a one hour labelling period was allowed; for $^{35}S$, the cells were labelled for 2 hours; and for $^{14}C$, the cells were grown to a density of $4 \times 10^8$ cells/ml before harvesting.

### D. Preparation and Purification of Phage T6

A culture of E. coli B, grown in H-broth using forced aeration to a density of $3 \times 10^8$ cells/ml was infected with phage T6 at a multiplicity of infection of 0.1. Aeration was continued while the infected culture

was allowed to lyse for 4.5 hours. Lysis was completed by the addition of chloroform (0.17 ml/l) during the last 10 minutes of incubation. The cell debris was removed by centrifugation at 10,000 g for 20 minutes, and the resultant crude lysate was assayed using the plaque assay method. This method consists of plating 1.0 ml of the appropriate phage dilution to which 0.2 ml of bacteria and 2 ml of molten T6 top agar was added on T6 hard agar plates. The plates were incubated overnight at 37° and scored for plaques. The phage was then sedimented in the Spinco Type 19 rotor at 18,000 rpm in the Beckman L2-65B ultracentrifuge. The resulting phage pellets were resuspended in M9 buffer at a concentration of $5 \times 10^{11}$ pfu/ml and clarified by centrifuging at 12,000 g for 10 min. Chloroform was added, after which the preparation was stored at 4°.

### E. Phage Spot Test

The sensitivity of a particular bacterial strain to bacteriophage T6 was tested by mixing 0.1 ml of bacterial culture with 3 ml of T6 top agar and spreading on a hard agar plate. A drop of phage lysate was placed on the plate and it was incubated at 37° overnight. Plaque formation was indicative of phage sensitivity.

### F. Assay for Radioactive Protein

Radioactively labelled *E. coli* preparations were assayed for TCA-insoluble material by applying aliquots of 20 to 100 μl onto 2.3 cm Whatman #3 filter discs pinned to a board. The paper discs were processed by a scheme modified from that of Mans and Novelli (1960), in which ethanol was used in place of ether for reasons of safety.

Preparation of Paper Discs for Hot TCA Insoluble Products

| Step | Time | Wash medium | Temperature |
|---|---|---|---|
| 1 | 15 min | 10% TCA | 4° |
| 2 | 5 min | 5% TCA | 4° |
| 3 | 45 min | 5% TCA | 90° |
| 4 | 15 min | 5% TCA | 4° |
| 5 | 15 min | 80% EtOH | 20° |
| 6 | 15 min | 95% EtOH | 20° |

The discs were then dried and placed in scintillation vials.

### G. Radioisotope counting

Dry samples such as air-dried filter discs were counted for 5 minutes in 5.0 ml of toluene-based scintillation fluid (prepared by adding 5.0 g PPO (2,5 diphenyloxazole) to 1.0 liter of scintillation grade toluene) in a Beckman LS-230 liquid scintillation spectrometer. This resulted in a counting accuracy of ± 3-5% for samples with low activity, and 0.2-1% for more active samples.

Single isotope restricted channels were used for double labelling experiments and counts were corrected for overlap.

All samples were corrected for background radioactivity by using controls which were treated the same, but containing no radioactivity.

### H. Sucrose Gradient Techniques

Linear (15 - 70%) 36 ml sucrose gradients were poured from a two-chamber Buchler gradient marker. After samples (0.5 ml) were layered onto the gradients, centrifugation was carried out at 6000 rpm and 20° for 15 minutes using the SW27 rotor in the Beckman L2-65B ultracentrifuge.

One ml fractions were collected by piercing the bottom of the tube with a hollow needle.

## I. Techniques Used in the Analysis of Proteins by Gel Electrophoresis

### 1. Two Dimensional Gel Electrophoresis

Two dimensional gel electrophoresis was performed on *E. coli* proteins according to the methods of O'Farrell (1975) and Ames and Nikaido (1976).

The first dimension, isoelectric focusing gel was prepared by mixing 9 M urea, 4% acrylamide, 2% nonident P40 (NP40) and 2% (w/v) ampholines. A 2:2:1 ratio of pH 4-6, pH 6-8 and pH 3.5 - 10 ampholines was used to give the pH range required. After degassing, 0.4 ml of a 0.14 mg/ml riboflavin solution containing 1% TEMED was added for polymerization. Gels were poured to a height of 12 cm in tubes having an inside diameter of 1.5 mm.

The protein was solubilized by heating 40 μl of protein solution with 26 μl of 10% (w/v) SDS (sodium dodecyl sulfate), 13 μl of 0.5 M Tris, pH 6.8, 1 μl of 0.1 M $MgCl_2$ and 2 μl each of 2.5 mg/ml DNase and RNase solutions, to 70° for 30 minutes. This was then centrifuged at 15,000 rpm for 90 minutes in the Sorvall RC2-B centrifuge. The supernatant solution was removed and diluted with 1 volume of sample dilution buffer (9.5 M urea, 2% (w/v) ampholines, 5% (v/v) β mercaptoethanol and 8% (v/v) NP40) before placing on the gel. 10 μl of sample overlay solution (4 M urea, 1% (w/v) ampholines) was layered on top. The gels were then run for 18 hours at 300 V, with 0.02 M NaOH (degassed) in the cathode reservoir and 0.01 M $H_3PO_4$ in the anode reservoir.

The second dimension consisted of SDS gel electrophoresis. The

running gel consisted of 10% acrylamide, 0.375 M Tris, pH 8.8, 0.1% LiDS (lithium dodecyl sulfate), and 1 mg/ml ammonium persulfate. A stacking gel consisting of 5% acrylamide, 0.375 M Tris, pH 8.8, 0.1% LiDS and 1 mg/ml ammonium persulfate was layered onto the running gel once the latter was polymerized.

For transfer from the first to the second dimension, the isoelectric focusing gel was carefully removed from its tube and transferred to the top of the stacking gel, where it was laid horizontally. To hold the gel in place, 0.5% agarose made up in running buffer was added and allowed to solidify. The running buffer consisted of 0.192 M glycine, 0.025 M Tris and 0.1% LiDS, pH 8.3. The gel was run at 25 mamps for one hour. The current was then increased to 50 mamps until the dye front reached the bottom of the gel.

### 2. Determining pH of IEF gels

Isoelectric focusing gels (IEF) which had had no sample applied to them were cut into 5 mm pieces and placed in test tubes to which 1 ml of degassed dionized water was added. After sitting for several hours, the pH of each tube was read using a pH meter (Radiometer, Copenhagen).

### 3. SDS Polyacrylamide Gel Electrophoresis

For one dimensional slab gel SDS polyacrylamide electrophoresis, a 12.5% gel containing 0.1 M phosphate, pH 7.0 and 0.1% SDS was used. The gel was polymerized with ammonium persulfate and TEMED. The running buffer consisted of 0.1 M phosphate, pH 7.0 and 0.1% SDS.

The protein samples were prepared in water and made 1% (w/v) in SDS, 1% (v/v) in β mercaptoethanol, and 0.08% (w/v) in sucrose. These were heated for 2 hours at 37°. Bromophenol blue was added as dye marker.

After the protein samples were loaded on the gel, it was run at a current of 30 mamps until the dye front reached the bottom of the gel. The gel was fixed in 10% acetic acid, 10% methanol overnight, then dried and exposed to X-ray film.

### 4. Counting Radioactivity in Gels

For counting the radioactivity in isoelectric focusing gels, the gels were sliced into 1.5 mm slices using a gel slicing block. Each slice was placed in a scintillation vial and to this was added 1 ml of an NCS-$NH_4OH$ (1:20 (v/v)) solution. The vials were capped and incubated overnight at 40°. The following day the vials were uncapped and incubated at 60° overnight. After the vials were allowed to cool, 5 ml of toluene-based scintillation fluid was added and the vials were counted for 5 minutes.

### 5. Treatment of Gels for Fluorography

When the protein to be run on a gel was labelled with $^3H$, the gels were treated for fluorography. The gels were first fixed overnight in 10% (v/v) acetic acid. They were then soaked in dimethylsulfoxide (DMSO) at room temperature. The DMSO was changed twice at 30 minute intervals. A solution of 20% (w/w) PPO in DMSO was poured over the gels and these were incubated for 3 hours at 25 - 30°. This solution was removed and the gels were rinsed with water. They remained in water overnight, before drying and exposing to X-ray film.

## J. Development of X-ray film

The film was placed in developer for 5 minutes, then rinsed in water for 30 seconds. It was then fixed for 7 minutes and allowed to rinse in water for 30 munutes before being dried.

# CHAPTER III

# RATIONALE OF THE EXPERIMENT

## A. Introduction

Any speculation on the role of F pili in bacterial conjugation must also take into account the mechanism by which F pili promote the infection of $F^+$ cells by male-specific bacteriophages. In this regard, a number of studies on F pilus-RNA phage interactions have suggested that the mechanism of RNA phage infection may be as shown schematically in figure 2. The A protein of the phage R17 serves as the organelle for attachment of the phage to F pili (Lodish *et al*. 1965; Heisenberg and Blessing, 1965; Curtiss and Krueger, 1974). Krahn *et al*. (1972) found that the A protein is transferred into the host cell along with the phage RNA. The suggestion from this that the A protein is physically linked to the phage RNA was subsequently confirmed by the demonstration (Wong and Paranchych, 1976) that the A protein is attached to the 3' end of the RNA, and that the A-protein-RNA complex is infectious (Liepold and Hofschneider, 1975; Reynolds and Paranchych, 1976). Interaction of the phage with the F pilus, which results in the cleavage of the 40,000 dalton A protein into two smaller peptides, with molecular weights of 25,000 and 15,000 daltons (Krahn *et al*. 1972), may be a signal which triggers the ejection of the A protein-RNA complex from the virion.

Penetration of the phage RNA into the host cell is sensitive to the action of ribonuclease (RNase). Also, approximately 50% of the RNA remains in a folded state during penetration (Wong and Paranchych, 1976). On the basis of these results, it was concluded that the A protein-RNA complex remains on the exterior surface of the F pilus

Figure 2 Proposed model for the mechanism of the adsorption and penetration of phage R17

A) The phage attaches to the F pilus via the A protein, which is physically linked to the 3' end of the RNA. B) The A protein is cleaved into two smaller peptides, triggering the release of A-protein-RNA complex from the phage capsid. C) The pilus retracts into the cell pulling with it the A protein-RNA complex. The empty capsid is then released.

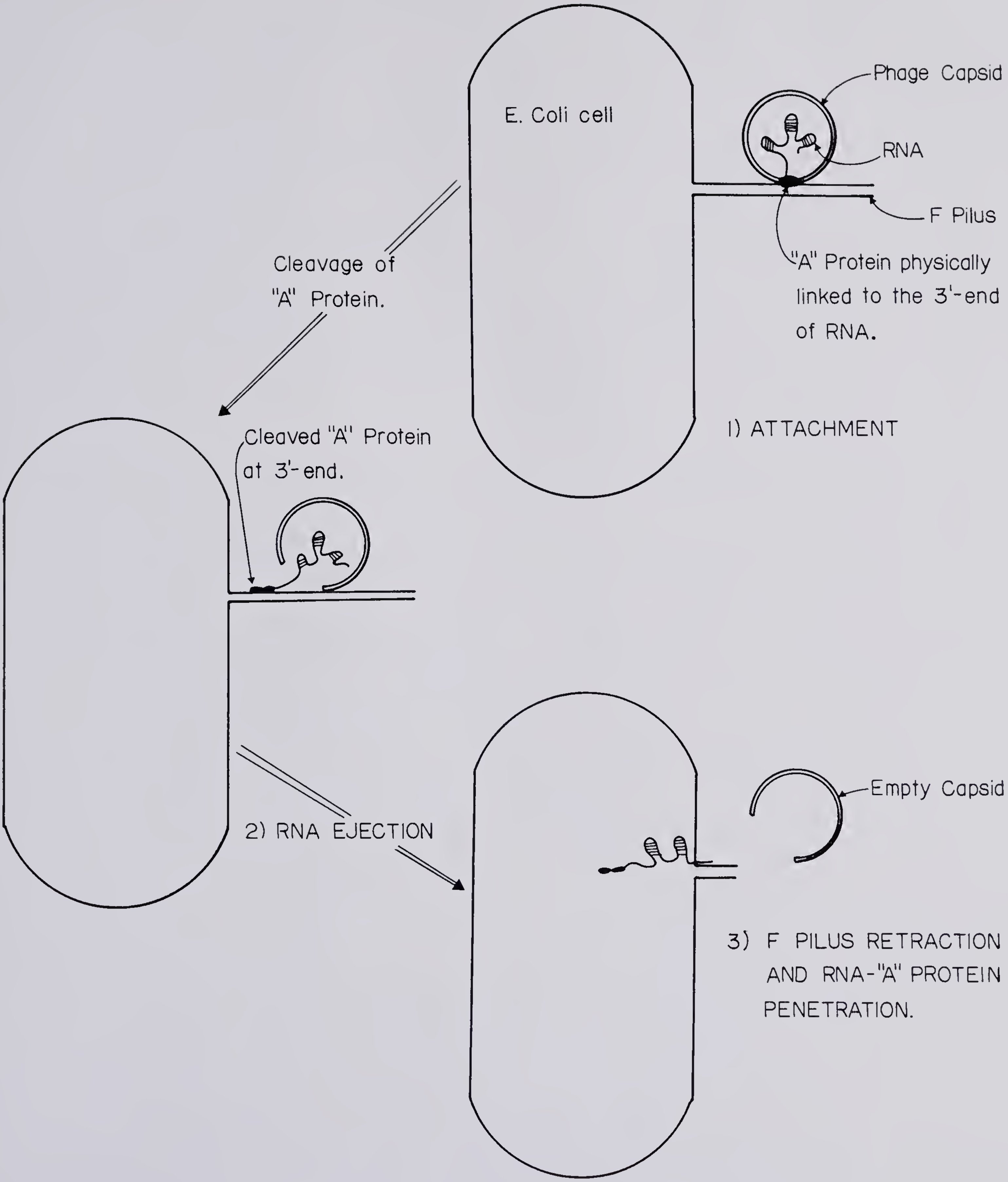
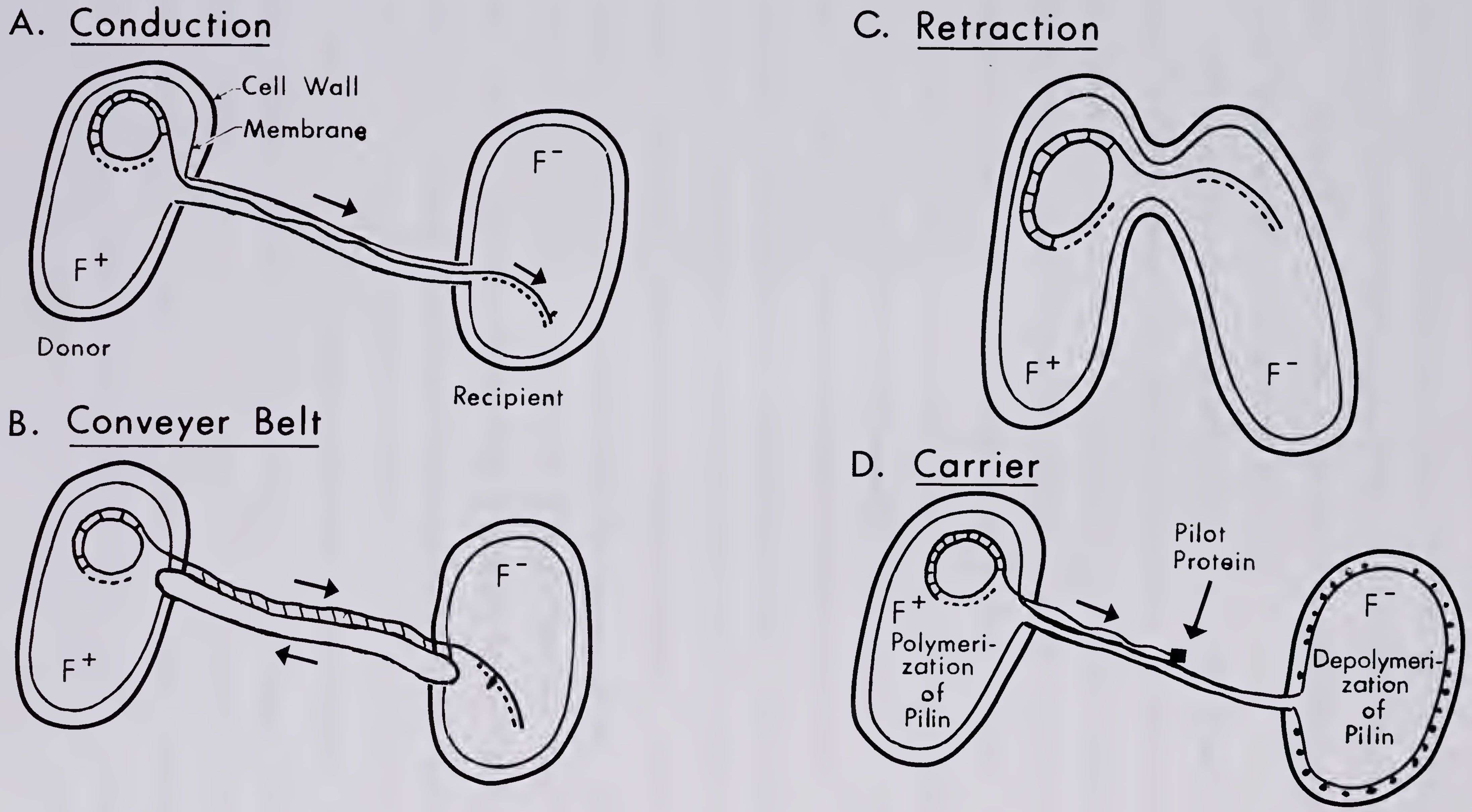

E. Coli cell
Phage Capsid
RNA
F Pilus
"A" Protein physically linked to the 3'-end of RNA.
1) ATTACHMENT
Cleavage of "A" Protein.
Cleaved "A" Protein at 3'-end.
2) RNA EJECTION
Empty Capsid
3) F PILUS RETRACTION AND RNA-"A" PROTEIN PENETRATION.

Figure 3 Models of the action of the F pilus in conjugation

A) Conduction model of Brinton (1965). The DNA is transferred through the hollow tube of the F pilus. B) Conveyor belt model of Brinton (1971). The two filaments of the F pilus move with respect to one another and transport the DNA to the recipient. C) Retraction model of Marvin and Hohn (1969) and Curtiss (1969). The pilus retracts into the donor cell when it contacts a recipient cell, bringing the two cells together. DNA transfer can then occur. D) Carrier model. Pilus subunits are polymerized in the donor cell membrane and depolymerized in the recipient cell membrane. A plasmid-specific pilot protein becomes anchored to the pilus and is transferred from the donor to the recipient cell along with the DNA.


A. Conduction
Cell Wall
Membrane
F+
F−
Donor
Recipient
B. Conveyer Belt
F+
F−
C. Retraction
F+
F−
D. Carrier
F+
Polymerization of Pilin
Pilot Protein
F−
Depolymerization of Pilin

during the process of ejection and penetration. Moreover, it was suggested (Wong and Paranchych, 1976) that the complex remains anchored to the pilus until retraction of the pilus brings the complex to the cell surface. The A protein then presumably pilots the RNA through the cell envelope into the cell.

This model, with minor modifications, can also be applied to explain the phenomenon of bacterial conjugation. Even before pili were discovered and known to function in conjugation, Anderson _et al_. (1957) proposed that DNA transfer between bacteria occurs via a conjugation bridge, which has since been called the "classical conjugation bridge". This was due to an electron micrograph showing a pair of mating cells connected by a slender bridge of material. Subsequent to this, F pili were recognized as being specific to $F^+$ cells and somehow involved in the conjugational process. The first model of how F pili function in conjugation was proposed in 1965 by Brinton (1965). This model and the other models of the role of F pili in conjugation are illustrated in figure 3. Brinton (1965) proposed that the pilus was a hollow tube through which the DNA was transferred from donor to recipient. There was no mention of how the DNA was transferred, whether there were any charge interactions between the protein of the pilus and the DNA passing through, or the nature of the driving force causing DNA movement. In 1971, Brinton (1971) changed his mind when he dissociated pili into filaments which were one-third the width of an intact pilus. Since he could also see a dark line down the center of intact pili in negative-stained preparations, he put forward a new proposal suggesting that pili may be composed of two rod-like filaments which move with respect to each

other to carry the DNA from donor to recipient in a conveyor belt mechanism. In neither of Brinton's models is there mention of a pilot protein, or of any type of protein transfer from donor to recipient during the conjugational process. Marvin and Hohn (1969) and Curtiss (1969) proposed that pili retract into the donor cell when they contact the recipient cell, bringing the two cells into close contact so that the membranes can fuse and DNA transfer can occur. Recently, Achtman (Achtman _et al_. 1978b) has shown that aggregation of cells occurs during mating and that extended pili can be dissolved by treatment with SDS without affecting mating. Although he mentions retraction of pili, he does not say that this is how the cells come into wall to wall contact nor does he state whether membrane fusion is an obligatory stage of the DNA transfer process. Based on the studies of phage R17 infection of _E. coli_, Paranchych has proposed the carrier model of conjugation in which F pilin subunits are transferred from donor to recipient as a result of assembly of pilin subunits in the donor cell membrane and the concomittant depolymerization of pilin subunits in the recipient cell membrane. Also included in this model is the idea that DNA transfer is achieved through the attachment of a plasmid-specific pilot protein to the 5' end (Ohki and Tomizawa, 1968; Ihler and Rupp, 1968) of the DNA.

These models would predict various patterns of protein transfer during conjugation. Neither the conduction nor the conveyor belt models would result in any protein transfer from donor to recipient cells. The retraction model, which involves a membrane fusion step, predicts that several donor cell proteins could be transferred to the recipient cell. Finally, the carrier model predicts that two proteins,

pilin subunits and a pilot protein, would be transferred to recipient cells during the conjugational process.

Examination of these various models reveals that it is theoretically possible to distinguish between them by determining how many donor cell proteins are transferred to recipient cells during bacterial mating. However, the feasibility of such an approach in practical terms is dependent upon whether it is possible to design an experimental protocol which allows the detection of low levels of donor cell proteins in isogenic recipients. Three previous attempts were made to examine protein transfer during conjugation. In 1959, Pardee, Jacob and Monod (Pardee _et_ _al_. 1959) used the induction of β-galactosidase synthesis to see if protein transfer, specifically that of the lac repressor, occurred during conjugation. Their conclusion that protein transfer does not occur is open to criticism since they were testing only for the transfer of functional lac repressor. Fisher (1962) looked for the transfer of the λ repressor as an indication of protein transfer during conjugation. He found that $F^-$ cells became immune to λ phage after conjugation with an $F^+$ cell which was carrying a lysogenized λ phage, indicating that protein transfer had taken place. However, if the donor was an Hfr cell, this effect of rendering the $F^-$ cells immune could not be seen. Pardee (Pardee _et_ _al_. 1959) had also used Hfr cells in his experiments. These results for $F^+$ and Hfr cells were not expected since this would indicate that the mechanisms of conjugation are different for $F^+$ and Hfr cells. In both these experiments, transfer of only certain proteins, that is the lac and λ repressors respectively, was tested. Silver (1963; Silver _et_ _al_. 1965) attempted to examine the overall transfer of proteins by mating

phage T6-resistant radioactively labelled donor cells with T6-sensitive recipient cells, then using lysis-from-without with T6 phage to kill the recipient cells. The mixture was then passed through a Millipore filter, and the filtrate, which should contain lysed recipient cells, was analyzed for radioactivity. Although it was concluded from the foregoing experiment that no conjugation-specific protein transfer occurred, control experiments showed that only half of the T6-lysed recipient cell material appeared in the filtrate. Thus, much of the recipient protein was retained on the filter and so was unavailable for the assay for radioactive counts. It has yet to be shown conclusively, therefore, whether protein transfer does or does not occur during conjugation.

It is evident from the foregoing discussion that the experimental protocol chosen to measure donor-to-recipient protein transfer must be capable of detecting exceedingly low levels of protein. For example, one F pilus (approximately 90 Å in diameter and 10,000 Å in length) would contain about $10^{-17}$ grams of pilus protein. Since this represents approximately 0.005% of the total cell protein, one would wish to ensure that the experimental procedure can (a) detect at least this amount of protein in the event that it is transferred to recipient cells, and (b) discriminate between this and a backgroud of donor-specific proteins which are not transferred during conjugation. Preliminary experiments described in the remainder of this chapter suggested that an experimental protocol can be devised to satisy these criteria. The procedure finally adopted involved the use of SDS-containing sucrose gradients to separate T6-lysed, radioactively labelled donor cells from T6-resistant, non-labelled recipient cells, poly-

acrylamide gel electrophoresis to separate bacterial proteins, and the careful choice of controls to distinguish between non-specific donor cell proteins and those which are specifically transferred to the recipient cell during bacterial mating.

## B. Results

Bacterial matings were carried out between phage T6-sensitive donor cells which had been radioactively labelled, and T6-resistant recipient cells which were not labelled. After a one hour mating period, in which the donor and recipient cells were mixed in a 1:1 ratio, the donor cells were killed by lysis-from-without with T6 phages. The T6-resistant recipients were then separated from the lysed material in a 15-70% linear sucrose gradient containing 0.1% SDS. Under these conditions, separation of the lysed from unlysed cells was at least partially achieved. The recipient cells were located by collecting 1 ml fractions from the gradients and reading the absorbance at 280 nm. The fractions containing the recipient cells were then pooled and centrifuged, after which the cell pellet was resuspended in water, lyophilized, and subjected to separation on polyacrylamide gels.

### 1. Studies on the Separation of Donor from Recipient Cells

In developing the foregoing procedure, the first problem to be examined was that of separating donor and recipient cells in a mating mixture. Lysis-from-without was chosen as the means of specificially killing donor cells while leaving the recipient cells intact. Fisher (1962) first used this procedure to separate donor from recipient cells in his experiments on the transfer of immunity to $\lambda$ phage

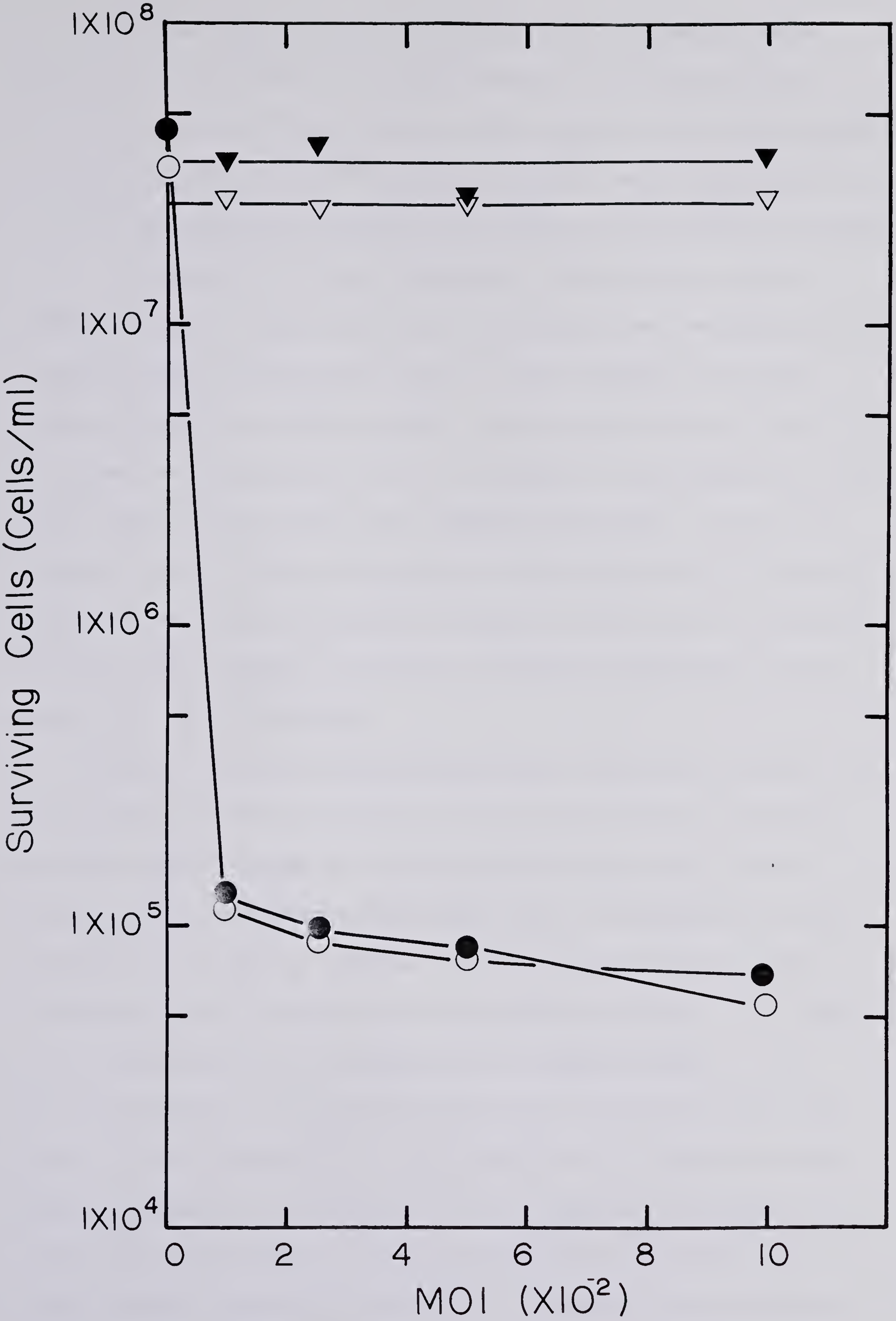
from donor to recipient cells.

To ensure that this procedure of T6 lysis of the donor cells would perform the designated task, and to determine the multiplicity of infection of T6 phages required for complete killing, the following experiment was performed. The four strains to be used in the mating experiments were grown to a density of 3 - $4 \times 10^7$ cells/ml, then challenged with various multiplicities of T6 phages. The phages were irradiated with UV light prior to adding to the cells in order to reduce the level of host range mutants which might attack the T6 resistant recipient strain. After a 30 minute incubation period, colony counts were performed to determine the number of surviving cells. The results of this experiment are shown in figure 4. The T6-resistant recipient strains, ED2601 and ED2602, are seen to be completely resistant to lysis by T6, whereas the T6 sensitive donor strains JC6583 and JC6256 are effectively killed, leaving just 0.2% of the cells surviving at a challenge multiplicity of 500 phages/cell. This multiplicity (500 phages/cell) was used in all subsequent experiments.

Following T6 treatment, the donor cells are present as cell ghosts and fragments, since lysis-from-without with T6 phages creates holes in the cell membrane, but does not necessarily destroy the cell entirely. Preliminary experiments aimed at the separation of the donor cell debris from intact recipient cells indicated that it could not be achieved by simple differential centrifugation or zonal centrifugation through sucrose gradients. It was presumed that pili were keeping the recipient cells attached to the donor ghosts and fragments. Since pili are readily dissociated by low concentrations of SDS, the effect of this detergent on the separation of recipient cells from donor cell debris

<u>Figure 4</u> Cell survival after incubation with T6 phages

Cells grown to a density of $3 \times 10^7$ cells/ml were infected with UV irradiated T6 phages at various multiplicities of infection. After a 30 minute incubation period colony counts were performed to determine the number of surviving cells. ▼ ED2601; ▽ ED2602; ● JC6583; o JC6256.

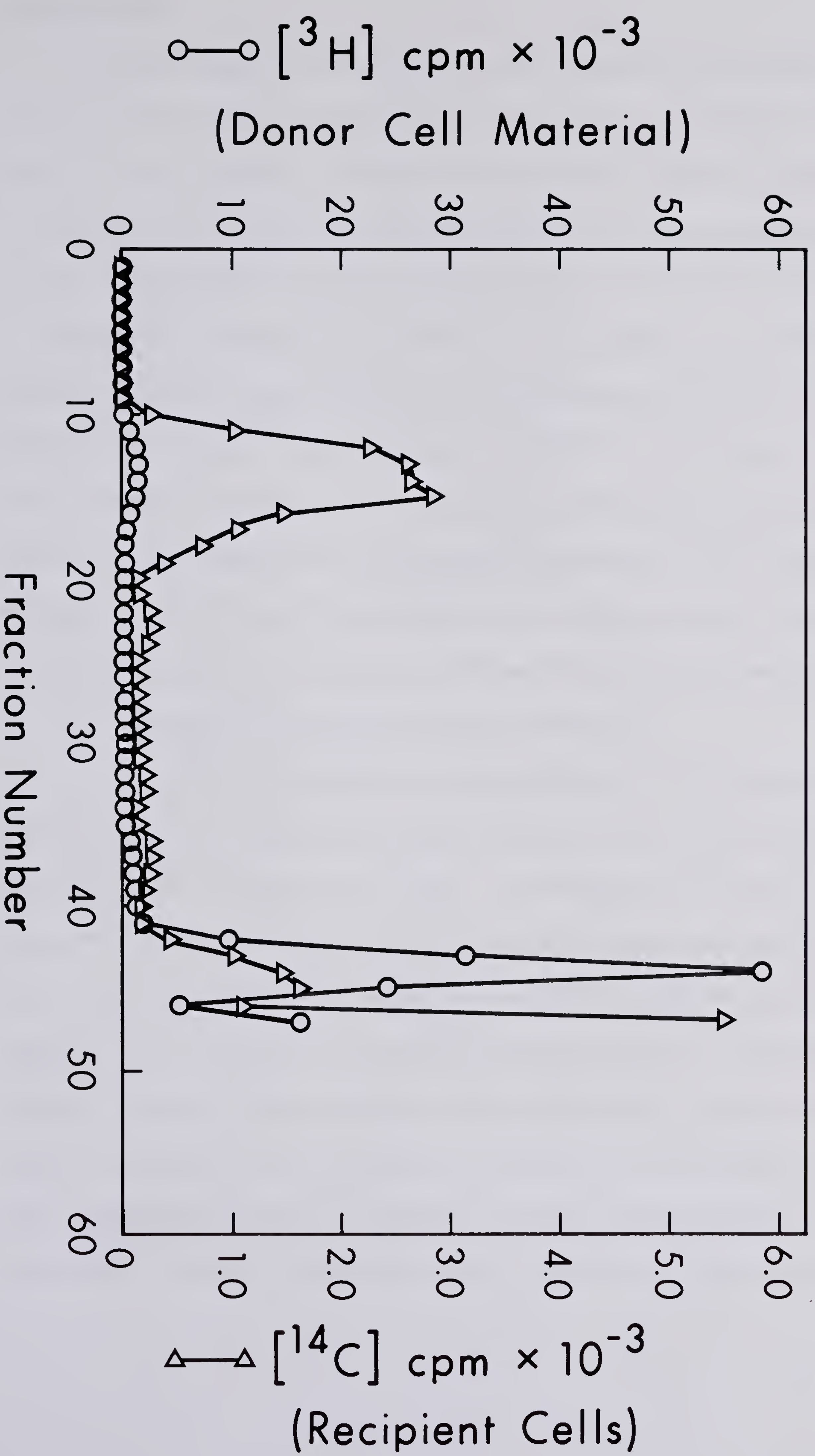

1X10⁸
1X10⁷
1X10⁶
1X10⁵
1X10⁴
Surviving Cells (Cells/ml)
0
2
4
6
8
10
MOI (X10⁻²)

was tested. As seen in figure 5, linear 15-70% sucrose gradients made up in 0.05 M Tris, pH 7.5 and containing 0.1% SDS effected a partial separation of the recipient cells from the lysed donor material. Approximately 30% of the $^{14}$C-labelled recipient cells sedimented free of the $^{3}$H-labelled donor material to a region of the gradient represented by fractions 9 -21 (fast sedimenting (FS) recipient cells). Repetition of this experiment four times showed that the proportion of recipient cells becoming free from the donor material in the SDS-sucrose gradient was highly variable, with a mean value of 27±13%. Use of higher concentrations of SDS did not improve this separation, while lower concentrations led to less effective separation. In fact, no separation was achieved at SDS concentrations below 0.01%. Thus about 70% of the recipient cell material remained at the top of the gradient with the donor material, or banded at densities intermediate between donor and recipient material.

There are several possible reasons why a considerable portion of the recipient material remains at the top of the sucrose gradients described above. Incomplete solubilization of the pili, or cell fusions which are SDS-resistant, would result in the donor and recipient material remaining together. Coating of the recipient cells with lipid material released from the lysed donor cells, could result in the cells banding in the gradient at a lighter density. Non-specific adhesion of donor and recipient cells would also have this effect. A final possibility is that the 30% of the recipient cells which do sediment in the gradient do so because they never came in contact with donor cells. However, this latter possibility is unlikely because no separation at all was effected by sucrose gradients

Figure 5 Separation of donor and recipient cells on a sucrose gradient containing 0.1% SDS

0.5 ml samples of the mixture containing the lysed donor material and intact recipient cells were layered onto a 36 ml 15-70% linear sucrose gradient containing 0.1% SDS. The gradients were centrifuged for 15 minutes at 6000 rpm in the SW27 rotor of the Beckman L2-65B ultracentrifuge. Approximately 0.5 ml fractions were collected and assayed for TCA precipitable radioactive counts.


[$^{3}$H] cpm × $10^{-3}$
(Donor Cell Material)
0
10
20
30
40
50
60
Fraction Number
0
10
20
30
40
50
60
0
1.0
2.0
3.0
4.0
5.0
6.0
[$^{14}$C] cpm × $10^{-3}$
(Recipient Cells)

lacking SDS.

The most likely explanation for the incomplete separation of recipient and donor cell material would appear to be that of non-specific cell adhesion. This was suggested by control experiments in which a $T6^S$ $F^-$ strain was substituted in the mating mixture in place of the $F^+$ donor strain. After lysis of the $T6^S$ $F^-$ cells with T6 phages and sedimentation of the $F^-$ x $F^-$ mixture in a SDS-sucrose gradient, it was found that the same proportion of $T6^R$ $F^-$ cells were trapped at the top of the gradient with $T6^S$ $F^-$ cell debris as in the case of the $F^+$ x $F^-$ mating. This result clearly implicated non-specific cell adhesion as the reason for recipient cell trapping, and suggested further that the analysis of the FS-recipient cell fraction for transfer-specific proteins would probably be experimentally valid.

### 2. Contamination with Donor Cell Proteins

The next problem to be considered was the possible contamination of the FS-recipient cell fraction with soluble proteins released from T6-lysed donor cells. Examination of figure 5 reveals that the $^{14}C$-labelled FS-recipient cell fractions were associated with a small amount of $^{3}H$ radioactivity from the donor cells (approximately 2.5% of the total donor cell radioactivity). To determine whether this low level of donor cell radioactivity represented non-specific contamination by proteins unrelated to the conjugation process, conjugation-specific transfer of donor cell proteins, or both, five types of control experiments were performed. These included:

1. $T6^S$ $F^+$ cells were lysed with T6 phages before $T6^R$ $F^-$ cells were added.
2. $T6^S$ $F^+$ cells were lysed with T6 phages before $T6^R$ $F^+$ cells were added.
3. $T6^S$ $F^-$ cells were lysed with T6 phages before $T6^R$ $F^-$ cells were added.
4. $T6^S$ $F^+$ cells and $T6^R$ $F^+$ cells were incubated together at 37° for 60 minutes, before being subjected to T6 phage treatment.
5. $T6^S$ $F^-$ cells and $T6^R$ $F^-$ cells were incubated together at 37° for 60 minutes, before being subjected to T6 phage treatment.

The actual mating experiment involved the incubation of $T6^S$ $F^+$ cells with $T6^R$ $F^-$ cells for 60 minutes at 37°, after which T6 phages were added for 30 minutes to cause lysis of the donor cells. In each case, the amount of donor cell radioactivity becoming associated with the FS recipient cell fractions was determined. The results of several such experiments are shown in Table 1. It may be seen that a small amount of donor cell radioactivity was found associated with the FS fraction in all of the control experiments, indicating that some contamination of recipient cells with donor cell material does occur. In general, $F^+$ x $F^+$ controls (2,4) resulted in less contamination of the FS fraction than $F^-$ x $F^-$ controls (3,5) or the $F^+$ x $F^-$ control (1). Although there was no statistically significant difference between several of the controls (1,3,5) and the actual mating experiment, the amount of donor cell radioactivity associated with the FS fraction for the mating experiment was nevertheless consistently higher than any of the controls. This suggested that the FS recipient cell fraction

Table 1

| Conditions of Mating Experiment | | Per Cent of Donor Cell Radioactivity (Hot TCA Insoluble) Found Associated with Fast Sedimenting Fraction[a] |
|---|---|---|
| Control 1. | $T6^S$ $F^+$ donor cells lysed with T6 then mixed with $T6^R$ $F^-$ cells | 2.1[b] ± 0.4[c] |
| Control 2. | $T6^S$ $F^+$ donor cells lysed with T6 then mixed with $T6^R$ $F^+$ cells | 1.4 ± 0.1 |
| Control 3. | $T6^S$ $F^-$ donor cells lysed with T6 then mixed with $T6^R$ $F^-$ cells | 1.9 ± 0.3 |
| Control 4. | $T6^S$ $F^+$ x $T6^R$ $F^+$, then lysis with T6 | 1.7 ± 0.2 |
| Control 5. | $T6^S$ $F^-$ x $T6^R$ $F^-$, then lysis with T6 | 2.2 ± 0.2 |
| Mating Experiment | $T6^S$ $F^+$ x $T6^R$ $F^-$, then lysis with T6 | 2.5 ± 0.3 |

---

[a] Fast sedimenting fraction was composed of those cells (fractions 12-30 of the sucrose gradient) which separated well from material at the top of the gradient

[b] Represents the mean of 11 separate experimental values

[c] Standard error of the mean

from an actual mating experiment may contain a small but detectable amount of conjugation-specific material in addition to the contaminating proteins released by T6 lysis of the donor cells. To determine whether conjugation-specific proteins could be detected in reality, the FS fractions were then subjected to polyacrylamide gel isoelectric focusing.

3. Analysis of Donor Cell Proteins Associated with Recipient Cells

The foregoing experiments involved the radioactive labelling of both donor ($^{3}$H-amino acids) and recipient ($^{14}$C-amino acids) cells in order to determine the distribution of each in SDS-sucrose gradients. However, to allow the detection of donor cell proteins by autoradiography, only donor cells were labelled in all subsequent experiments. The radioactive label normally used was $^{35}$S, as $Na_2{}^{35}SO_4$, since this gave the highest specific activity of the proteins. In these experiments, the FS fraction was located by determining the absorbance at 280 nm of each of the fractions collected from the SDS-sucrose gradients. These fractions were then pooled, diluted with water and the cells were sedimented by centrifuging for 2 hours at 23,000 rpm in the SW27 rotor of the Beckman L2-65B ultra-centrifuge. This cellular protein was then lyophilized in preparation for running on polyacrylamide gels.

Polyacrylamide isoelectric focusing (IEF) was chosen for the initial examination of recipient cell proteins because of its high resolving power for cellular proteins of _E. coli_. The initial problem encountered in setting up the IEF system was the lack of being able to produce a good pH gradient. Various combinations of ampholytes were used, but invariably a plateau extending over half the gel was present

in the basic pH range, as seen in figure 6A. Also in these cases, the change in pH from top to bottom of the gradient was usually about 2 pH units. To overcome these difficulties, time course experiments, in which tube gels were removed from the electrophoresis chamber and sliced for pH determination at times ranging from 1 to 24 hours, were performed and various combinations of ampholytes were used to determine which conditions would give the best pH gradient. It was finally decided to use the mixture of pH 4-6, pH 6-8 and pH 3.5-10 ampholytes in a 2:2:1 ratio at a total concentration of 2% ampholytes in the gel, and to run the gels for 18 hours at 300 V. The gradient obtained under these conditions, shown in figure 6B, had pH range of about 4.0 to 8.3, with some variation of the top and bottom limits in different experiments. The biphasic nature of the pH gradient was normally seen, with a shallow slope over most of the gradient, and a steeper slope in the region of the low pH values.

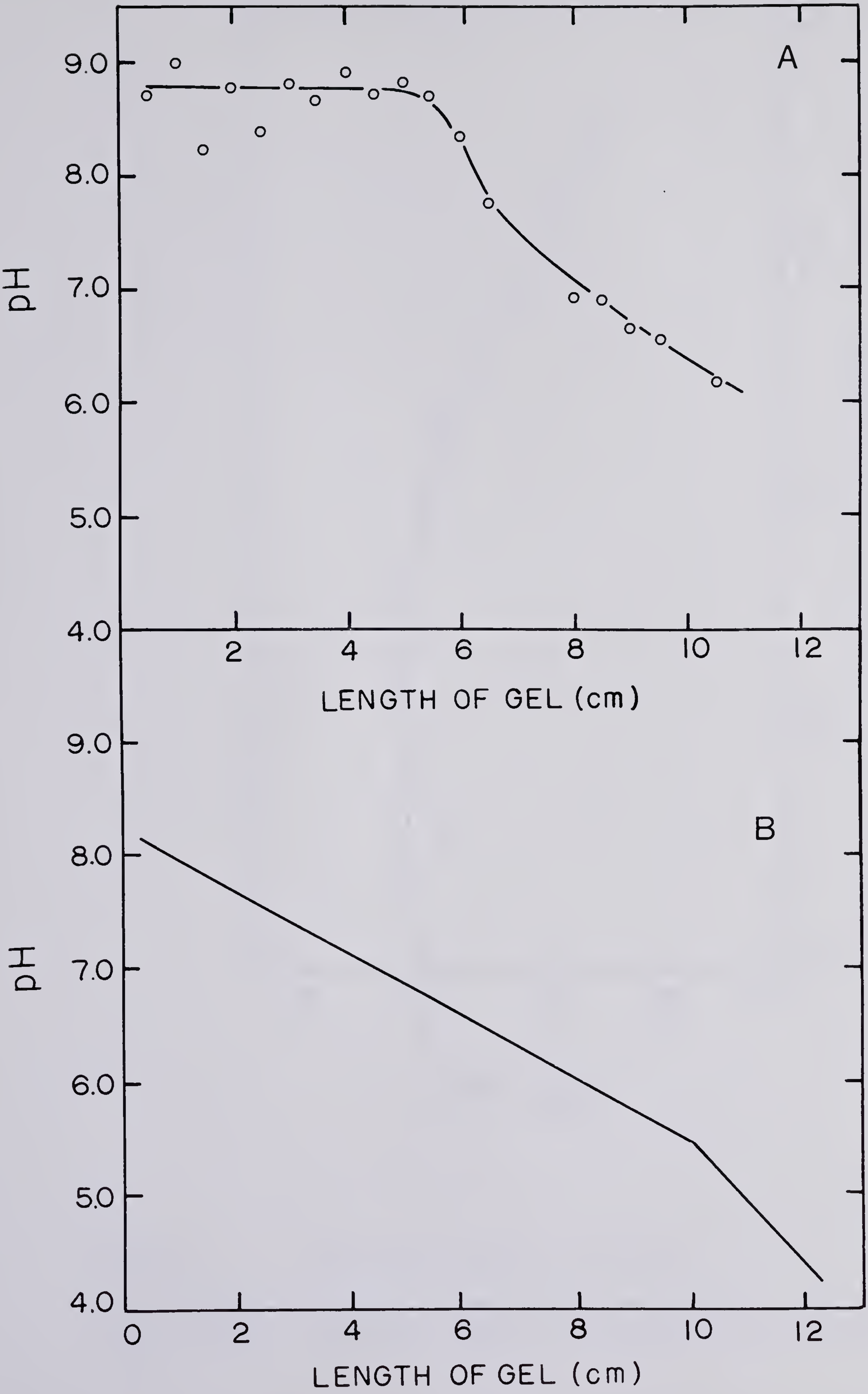
The IEF gels on which samples were run were analyzed in one of two ways. They were either dried and exposed to X-ray film for autoradiography, or they were sliced into small pieces and the radioactivity in each slice was counted. When the latter method was used, blank IEF gels were run and sliced along with the samples from the mating experiment and the controls, in order to determine the background radioactivity of the gels. As seen in figure 7, the background was constant at about 65 cpm over the first half of the gel, but then decreased to 30 cpm at the bottom of the gel. This background pattern may be due to focusing of naturally occurring isotopes in the top half of the IEF gel.

Preliminary results from the IEF gels which were sliced and counted

Figure 6 pH gradient of isoelectric focusing gels

A) The ampholytes in this gel were a 4:1 ratio of pH 5.7 and pH 3.5-10 ampholytes, respectively, to a total concentration of 2.0%. The gel was run for 20 hours at 300V. The pH of 5 mm slices of the gel was determined by soaking the slice in 1 ml of water for several hours before reading the pH.

B) The ampholytes in this gel were a 2:2:1 ratio of pH 4-6, pH 6-8 and pH 3.5-10 ampholytes, to a total concentration of 2.0%. The gel was run for 18 hours at 300V. This combination of ampholytes was used in all subsequent experiments.


A
pH
9.0
8.0
7.0
6.0
5.0
4.0
2
4
6
8
10
12
LENGTH OF GEL (cm)
B
pH
9.0
8.0
7.0
6.0
5.0
4.0
0
2
4
6
8
10
12
LENGTH OF GEL (cm)

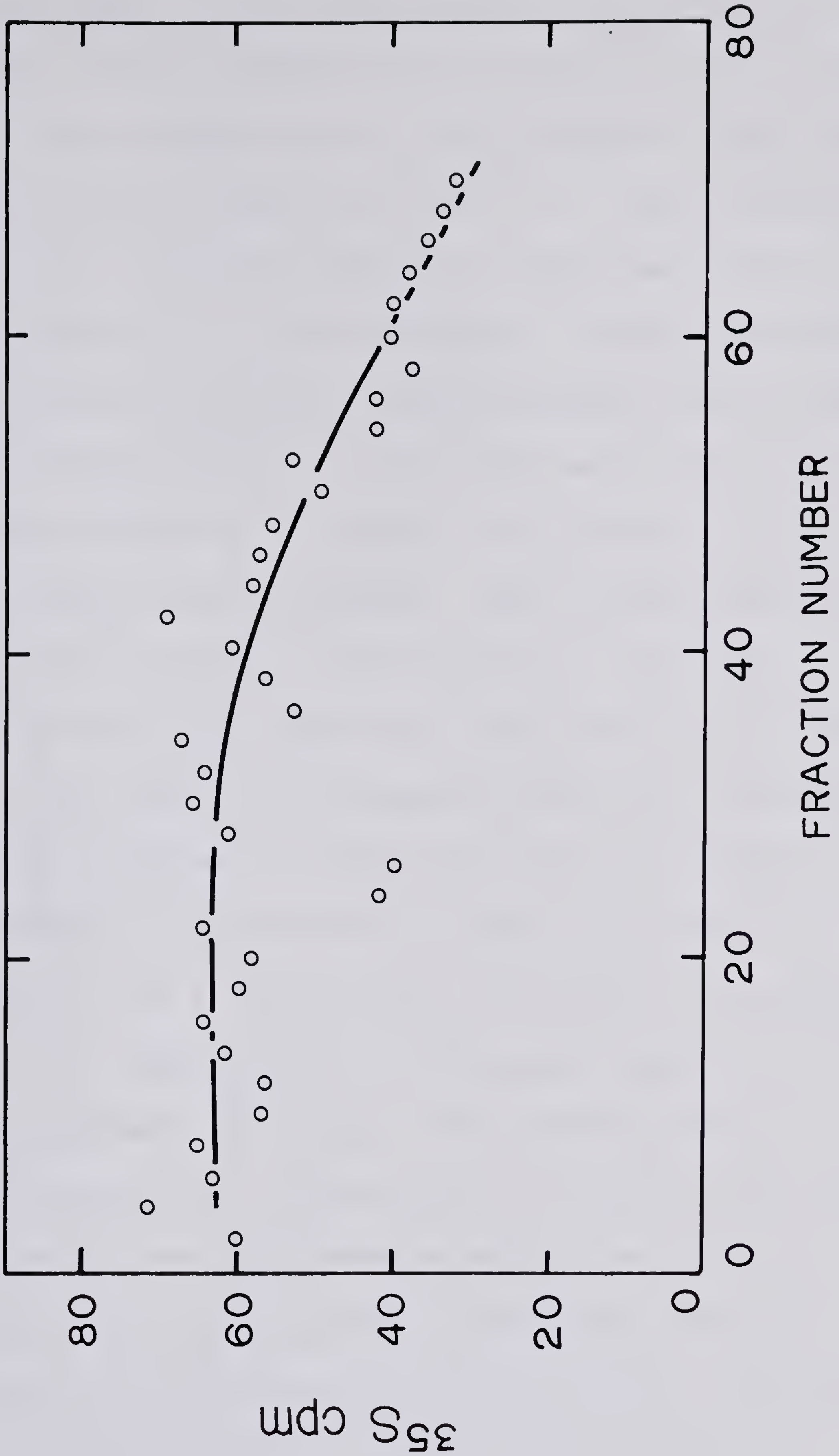


Figure 7 Background cpm for an IEF gel

The background radioactivity was determined by slicing and counting blank IEF gels. The curve shown in the figure is the average of 6 gels.

showed that several donor proteins were associated with the FS fraction of recipient cells. The gel profile of the average of 2 $F^+ \times F^-$ mating experiments, shown in figure 8 is a typical example. Comparison of this with the profiles of the $F^+ \times F^+$ and $F^- \times F^-$ control experiments indicated that no proteins specific to conjugation could be detected on the profile of the mating experiment. However, this does not necessarily mean that conjugation-specific protein transfer does not occur. The protein bands are very sharp and can be very close together, so that two bands may be present on one gel slice, thus showing up as one peak on the gel profile. Also, protein bands may be split by the slicing technique, thus appearing as two peaks.

The autoradiograms of gels which were dried gave some indication that protein transfer specific to conjugation does occur. Autoradiograms of the mating experiment plus the five controls are shown in figure 9. Unfortunately, these are not as clear as was hoped for. This could possibly be due to the fact that the isoelectric focusing gels are difficult to handle because of their small diameter and low concentration of acrylamide in the gel. This made them difficult to line up and keep straight when they were dried. Because of the background shadows, analysis of these autoradiograms proved difficult. Controls 1 ,2 and 3, shown in figure 9B, C and D had no detectable bands. A few bands could be detected in controls 4 and 5, figure 9E and F, but these could be matched to those detected in the actual mating, figure 9A. In spite of the difficulties involved in analyzing these gels it was possible to identify two bands in the autoradiogram of the actual mating experiment, with isoelectric points of 7.0 and 6.3, which appear to be unique to the $F^+ \times F^-$ mating.

Figure 8 Distribution of $^{35}S$ radioactivity in an IEF gel of recipient cells from an $F^+$ X $F^-$ mating experiment.

The mating experiment was performed as described in the text. After subjecting the purified recipient cell extract to IEF, the gel was sliced into 1.5 mm slices and assayed for radioactivity as described in Materials and Methods. The results are a composite of two separate gels.

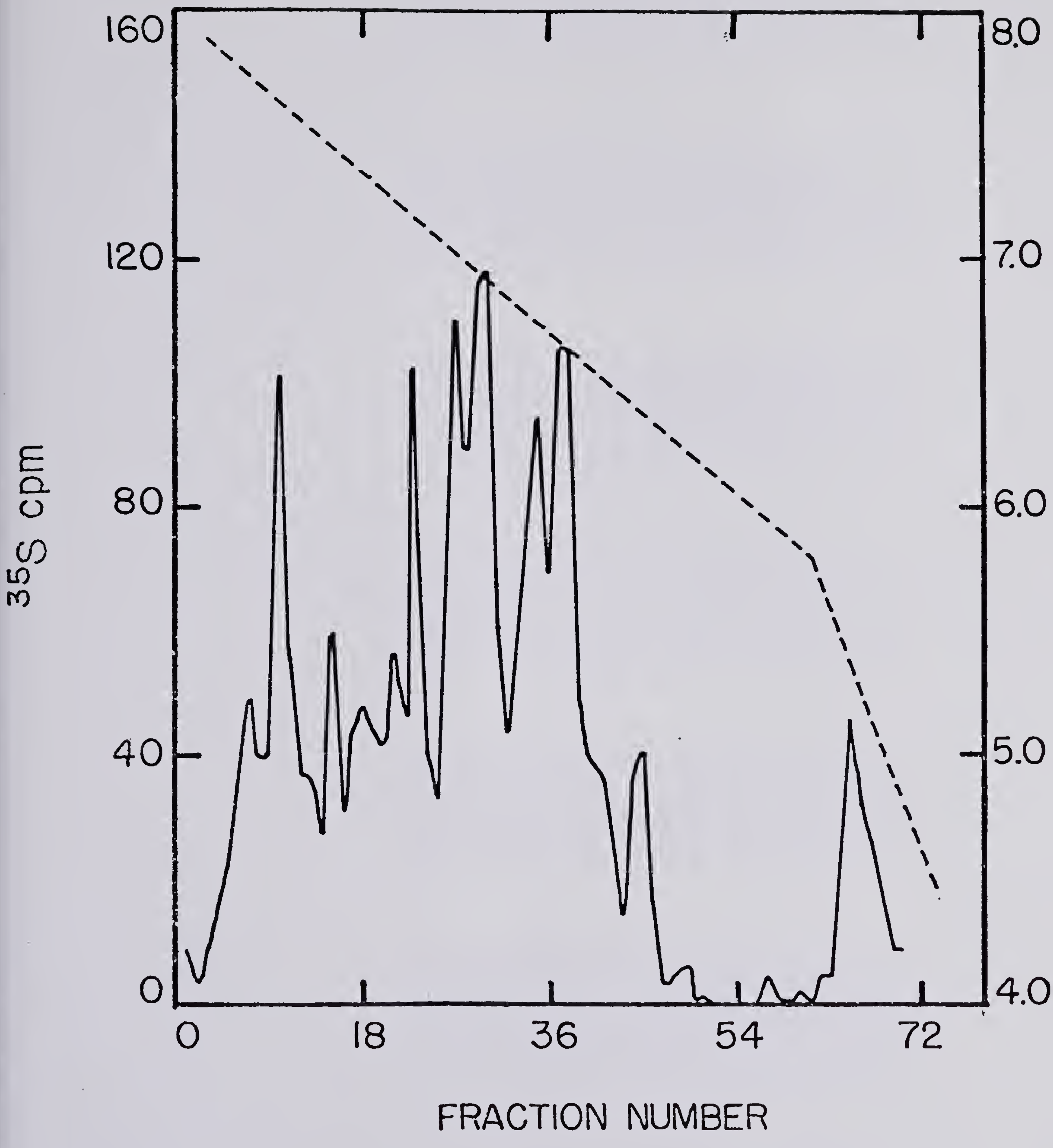
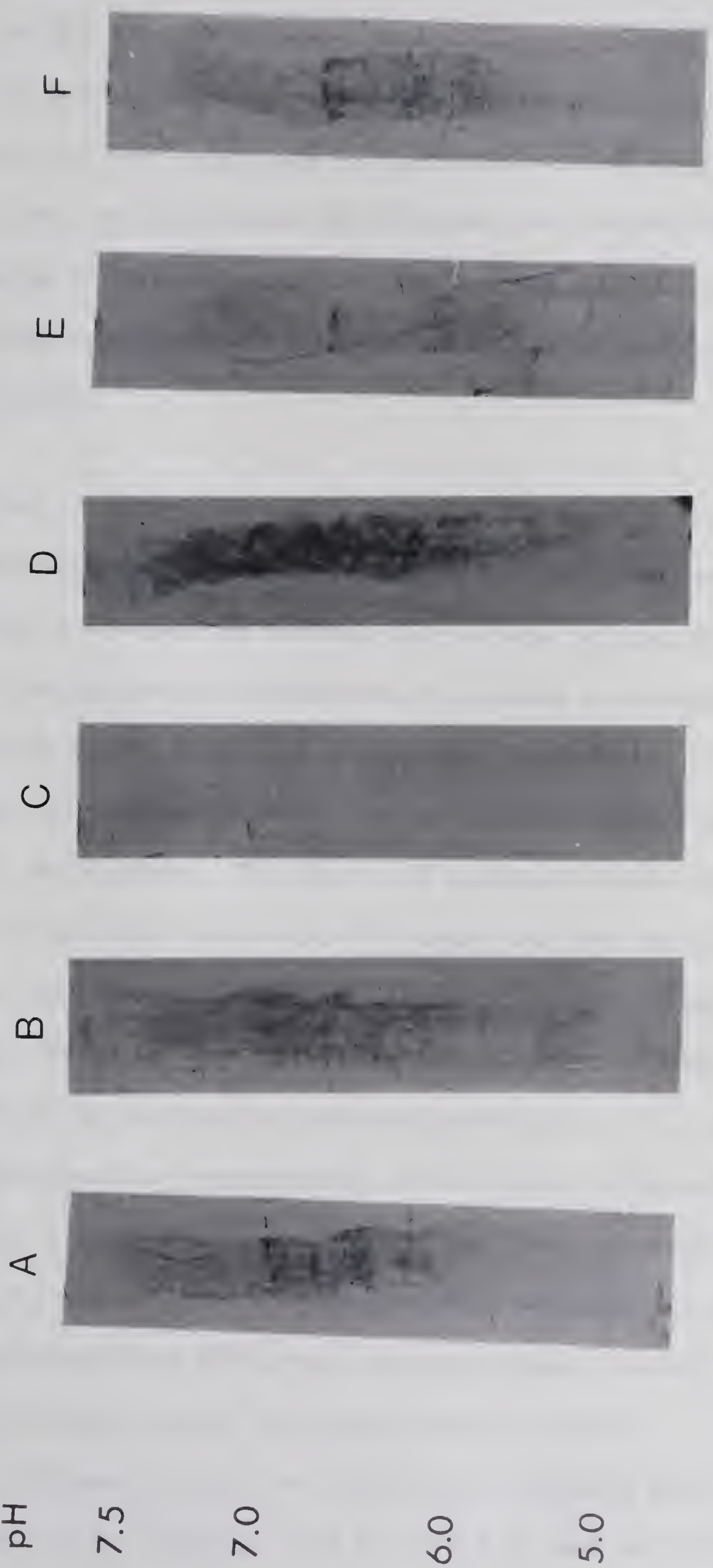

160
120
80
40
0
$^{35}S$ cpm
8.0
7.0
6.0
5.0
4.0
pH (----)
0
18
36
54
72
FRACTION NUMBER

Figure 9 Autoradiograms of IEF gels

These autoradiograms of the IEF gels from a mating experiment show some differences between the actual mating experiment and the controls. A) Mating experiment, $F^+$ $T6^S$ x $F^-$ $T6^R$; B) Control 1, $F^-$ $T6^R$ added to $F^+$ $T6^S$ after lysis with T6; C) Control 2, $F^+$ $T6^R$ added to $F^+$ $T6^S$ after lysis with T6; D) Control 3, $F^-$ $T6^R$ added to $F^-$ $T6^S$ after lysis with T6; E) Control 4, $F^+$ $T6^S$ x $F^+$ $T6^R$, then lysis with T6; F) Control 5, $F^-$ $T6^S$ x $F^-$ $T6^R$, then lysis with T6.


pH
7.5
7.0
6.0
5.0
A
B
C
D
E
F

Because of the difficulties inherent in the techniques of analyzing the IEF gels, which make it difficult to arrive at conclusive results, and since two or more proteins may have the same isoelectric point so they would not be separated in these one dimensional IEF gels, it was decided to use the O'Farrell two dimensional electrophoresis system (O'Farrell, 1975) to further separate the proteins from the mating experiments. These results will be discussed in the following chapter.

## C. Discussion

The mating experiments described in this chapter were designed to try to make a distinction between the various models of F pilus function in conjugation by determining if and how many donor proteins are transferred to the recipient during conjugation.

A number of problems inherent in the experiment were solved as described in the chapter. The donor and recipient cells were separated by lysing the donor cells with T6 phages and then spinning the mixture in an SDS sucrose gradient. Although complete separation was not achieved, enough of the recipient cells sedimented free of donor cell material to be analyzed on polyacrylamide gels. To overcome the problem of non-specific contamination of recipient cells with donor cell material, a number of controls were run. Any proteins appearing in the controls would be due to non-specific contamination and these could be eliminated from the actual main experiment leaving only the proteins which were a result of conjugational transfer.

The best pH gradient for the isoelectric focusing gels could be obtained with a 2:2:1 mixture of pH 4-6, pH 6-8, and pH 3.5-10 ampho-

lytes at a concentration of 2% in IEF gels which were electrophoresed for 18 hours at 300 V. Two bands, with isoelectric points of 7.0 and 6.3, were detected as being unique to the actual mating experiment. However, due to the large amount of background radioactivity exposing the film, and the lack of any results from the gel profiles of the IEF gels, two dimensional electrophoresis, as described in the following chapter, was used to further separate the recipient cell proteins.

# CHAPTER IV

# RESULTS AND DISCUSSION

## A. Introduction

The mating experiments described in the previous chapter were undertaken to determine if a distinction could be made between the various models of the role of F pili in bacterial conjugation, on the basis of whether or not protein transfer occurs from donor to recipient. After having examined several of the problems encountered in the experimental protocol, the final experimental procedure adopted was as follows. T6-sensitive, radioactively labelled donor cells were mated for one hour with T6-resistant non-labelled recipient cells, after which the donor cells were killed by lysis-from-without with T6 phages. The intact recipient cells and donor cell debris were pelletted, resuspended in a small volume of L-broth containing 0.1% SDS, and then the recipient cells were separated from the donor debris by centrifuging in a linear 15-70% sucrose gradient containing 0.1% SDS. The fractions containing the recipient cells, which were located by determining the absorbance at 280 nm of each of the fractions, were pooled and the cells sedimented and lyophilized. The recipient cell proteins were then solubilized in preparation for running on polyacrylamide gels.

## B. Results

Because the results from the isoelectric focusing gels were somewhat ambiguous, it was decided to use the O'Farrell two dimensional electrophoresis system (O'Farrell, 1975) to achieve resolution of cell

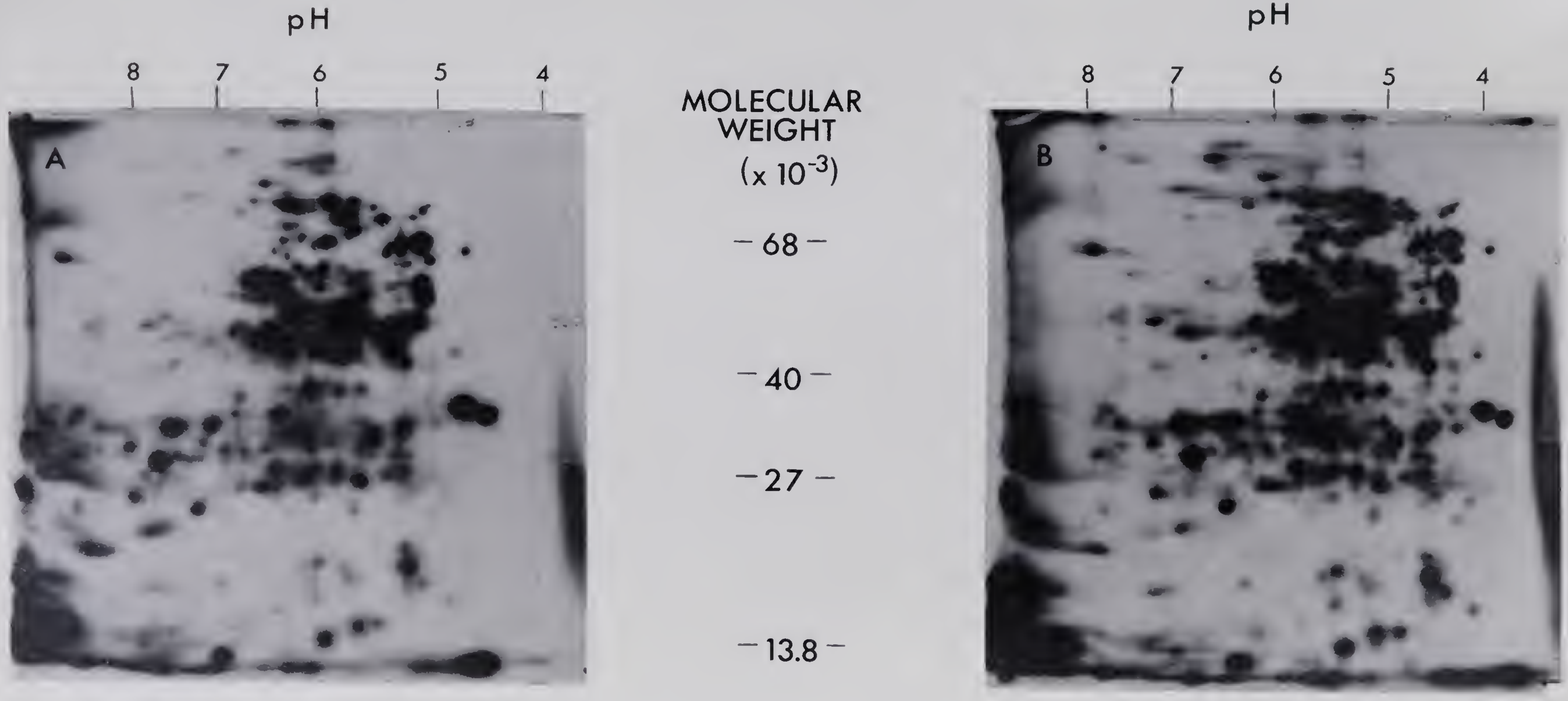
proteins in the mating experiment. This system separates proteins on the basis of isoelectric point in the first dimension, and according to molecular weight in SDS gels in the second.

The first dimension isoelectric focusing gel was prepared and electrophoresed in the same manner described in the previous chapter. This IEF gel was then laid horizontally across the top of the SDS gel and the proteins were electrophoresed in the second dimension at 50 mamps until the bromophenol blue dye front reached the bottom of the gel. To ensure that the two dimensional system was working, whole cell proteins of an $F^+$ (ED2602) and $F^-$ (ED2601) strain of _E. coli_ were electrophoresed. In figure 10, autoradiograms of these gels show that approximately 300-400 protein spots can be clearly detected. A majority of the proteins are concentrated in the middle of the gel, between pH 5 and 7, and molecular weights 40,000 to 60,000, so that considerable overlap occurs in this area. This region is also overexposed in these autoradiograms, but this was necessary in order to visualize the spots which were of lower intensity. Autoradiograms which look like the ones seen in the literature could be obtained by shorter exposure times. This system was then used to separate radioactive donor cell proteins associated with recipient cells following bacterial mating.

Most of the protein peaks and bands which were seen in the single dimension IEF gels disappeared when the proteins were separated in two dimensions. As may be seen in figure 11, no spots were seen in any of the control experiments, while just three spots were observed on autoradiograms of the actual mating. One possible reason for this may be that isoelectric focusing produces very sharp and distinct bands that

Figure 10 $F^+$ and $F^-$ whole cell proteins of *E. coli* K12.

Strains ED2601 ($F^-$) and ED2602 ($F^+$) were grown to $2\times10^8$ cells/ml in minimal medium containing the required amino acids and inorganic $^{35}SO_4$. the cells from each culture were then washed and subjected to two-dimensional polyacrylamide gel electrophoresis according to O'Farrell (1975) except that the pH gradient used in the isoelectric focusing step was 4-9. After electrophoresis, the gels were dried on a Bio-Rad drying apparatus and exposed to X-ray film for autoradiography.



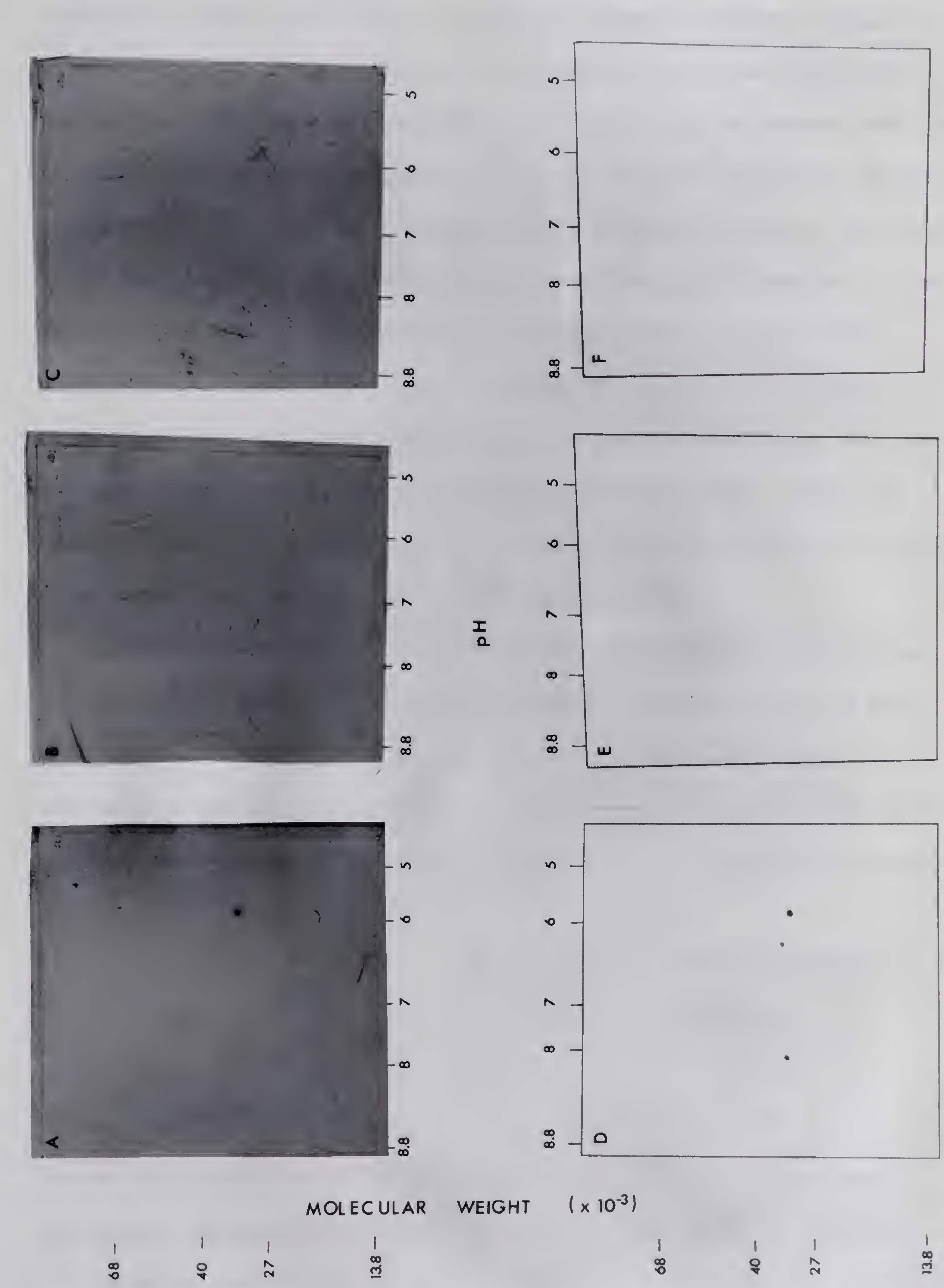
ED2601

ED2602

Figure 10

Figure 11 Autoradiographs of recipient cells obtained from $F^+$ x $F^-$, $F^-$ x $F^-$ and $F^+$ x $F^+$ matings with various strains of *E. coli* K12

$T6^r$ recipient cells were incubated for 60 minutes at 37°C with an equal number of $^{35}S$ labelled $T6^s$ donor cells. The recipient cells were then separated from mating aggregates by centrifugation through a linear 15-70% sucrose gradient containing 0.1% SDS, and subjected to two-dimensional polyacrylamide gel electrophoresis and autoradiography. These autoradiograms are examples of ones obtained in several experiments. Autoradiograms of (A) $F^+$ x $F^-$, (B) $F^-$ x $F^-$ and (C) $F^+$ x $F^+$ are shown in top half of Figure. (D) Schematic of A, (E) Schematic of B, (F) Schematic of C.


A
B
C
D
E
F
5
6
7
8
8.8
pH
MOLECULAR WEIGHT ( x 10-3)
68
40
27
13.8

can be detected at very low protein concentration. Since the second dimension causes these bands to diffuse in two directions during the electrophoresis, the protein is now not as concentrated, and there may not be sufficient radioactivity in a small area to expose the film. However, leaving the gels exposed to X-ray film for very long periods of time failed to reveal a larger number of protein spots on the film. It is also possible that the proteins from the first dimension do not penetrate the second dimension gel, although proteins were never detected at the top of the second dimension gel. Even in whole cell protein preparations of _E. coli_ cells, as depicted in figure 10, few proteins remain at the top of the second dimension gel. Therefore, non-penetration of the protein is probably not the cause for the loss of protein from the first to the second dimension.

Three protein spots were detected on the autoradiograms of the actual mating cross, $T6^S$ $F^+$ x $T6^R$ $F^-$ cells. Autoradiograms of this plus the two controls $F^-$ x $F^-$ and $F^+$ x $F^+$ are shown in figure 11. Schematic diagrams of the gels are drawn below each of the autoradiograms. These spots, from right to left, had the following characteristics:

| | pI | Molecular Weight |
|---|---|---|
| Spot 1 | 5.8 | 33,400 |
| Spot 2 | 6.2 | 34,300 |
| Spot 3 | 7.9 | 33,600 |

The pI was read from the pH gradient of the IEF gel of the first dimension, which was determined by slicing blank gels and reading the pH after soaking the slices in water for several hours. Molecular weight standards were run for the second dimension SDS gel in order

to determine the molecular weights of the proteins. Spot 1 (pI 5.8, MW 33,400) was the major spot appearing on the autoradiograms, whereas the other two were much fainter, with Spot 3 (pI 7.9, MW 33,600) having the next highest intensity.

F pilin was one of the proteins which was specifically being looked for in all the gels - unfortunately, it was never detected. Pilin has a molecular weight of about 12,000 in SDS gels and an isoelectric point of 3.5 to 4.15 (Valentine *et al*. 1969; Brinton, 1971; Beard *et al*. 1972; Date *et al*. 1978). It is therefore questionable whether such an acidic protein would penetrate into IEF gels whose pH range only occasionally extends as low as pH 4.0. It was also suspected that pilin may not penetrate the SDS gel, since pilin is difficult to solubilize sufficiently to penetrate one dimensional SDS gels. To determine if either of the foregoing possibilities may be correct, some pure F pilin was electrophoresed in the two dimensional system and the gel was stained with Coomassie blue to locate the protein. At the same time, a single dimensional IEF gel was also run. The pilin did penetrate the IEF gel and focused as a band near the bottom of the gel, at about pH 4.5. However, a spot corresponding to pilin could not be detected on the second dimension SDS gel, indicating a lack of penetration into the SDS gel. Because F pilin could not be visualized on the two dimensional gels, a one dimensional SDS polyacrylamide gel electrophoretic run was performed to determine whether pilin could be detected in this manner. The samples for the one dimensional SDS gel electrophoresis were prepared as described in Chapter II and electrophoresed in a phosphate buffer system. These gels were

dried and exposed to X-ray film after the electrophoresis to obtain autoradiograms. No conclusions could be drawn from these gels since no bands were detected in the gel, except for one at the very top of the gel which would indicate that the protein was unable to penetrate the gel.

## C. Dicussion

The mating experiments developed and performed in this study were done in the hope of being able to make a distinction between the various models for the role of F pili in conjugation by determining whether any protein transfer from donor to recipient cell occurs during the conjugational process. The occurrence of protein transfer, as detected on polyacrylamide gels, has allowed a distinction to be made between the four proposed models.

Although radioactive profiles of isoelectric focusing gels obtained by counting gel slices yielded no information about the transfer of plasmid-specific proteins during bacterial conjugation,the autoradiograms of the IEF gels indicated that two bands, with isoelectric points of 7.0 and 6.3 may be unique donor-specific proteins. Two dimensional electrophoresis yielded three protein spots which were unique to the actual mating experiment. The most intense spot had a pI of 5.8 and a molecular weight of 33,400; the next most intense spot had a pI of 7.9 and a molecular weight of 33,600; and the weakest spot had a pI of 6.2 and molecular weight of 34,300. A comparison of these results shows the band of pI 6.3 from the IEF gels could correspond to the spot in the two dimensional gels which has a pI of 6.2. The other two spots from the two dimensional gels could not be found on the IEF gels and

the second band of the IEF gels could not be detected on the autoradiograms of the two dimensional gels. Pilin could not be detected in either system. This does not necessarily mean that pilin is not transferred during conjugation process. Pilin is notoriously susceptible to aggregation in SDS solutions and is difficult to detect in polyacrylamide gel systems. From the polyacrylamide gel results, it would appear that possibly three proteins are transferred from the donor to the recipient cell during conjugation.

The foregoing results show that conjugation-specific protein transfer does occur during bacterial mating. This would suggest that the conduction (1965) and conveyor belt (1971) models of Brinton are unlikely mechanisms for F pili function in conjugation. These models predict that only DNA is transferred from donor to recipient via the F pilus. On the other hand, both the retraction (Curtiss, 1969; Marvin and Hohn, 1969) and carrier models predict that protein transfer should occur. In the retraction model, the F pili retract, bringing the cells into wall to wall contact with fusion of cell envelopes. This could result in the transfer of several proteins from donor to recipient cells. The carrier model predicts the transfer of two proteins, F pilin subunits and a carrier protein. Some support for the carrier model, and against the retraction model was provided in 1971 by Salzman (1971), who showed that blocking the tip of the F pilus with an F-specific filamentous phage in a cell carrying plasmid which directed the synthesis of both F and I type pili specifically blocked transfer of the F plasmid to a greater extent than the R plasmid specifying the I type pilus. Although the reverse was also true, the amount of transfer of the plasmid not blocked by the phages was

also decreased significantly, to about 35% of normal. The inhibition of transfer for the plasmid blocked by the phage was about 90%. This argues against the retraction model since both plasmids should be transferred efficiently through a conjugation bridge unless specific proteins are involved in the transfer of each plasmid. Moreover, the formation of a conjugation bridge between donor and recipient cells would presumably involve the exchange of more than three proteins in the cell envelopes. In the case of the carrier model, it was predicted that two proteins, a pilot protein and pilin should be transferred to recipient cells. Unfortunately, the experimental procedures were unable to determine whether pilin was one of the transferred proteins. Of the three proteins that are transferred to recipient cells, however, none corresponds precisely to any known plasmid specific proteins encoded by the *tra* operon. Kennedy *et al*. (1977) have used chimeric plasmids carrying EcoR1 fragments of the *tra* operon to identify and assign proteins encoded by *tra* genes. The molecular weight values obtained by these workers are shown in Table 2.

It is evident that the molecular weights of the three detected proteins do not match those of any of the *tra* gene products. However, allowing for differences in the electrophoresis systems, and therefore possibly different values for the molecular weights, it is possible that the gene products of *tra*B (MW 29,000), *tra*F (MW 25,000), *tra*T (MW 25,000) or *tra*H (MW 40,000) could be the same as one or more of the detected proteins. The three genes *tra*B, *tra*F and *tra*H are involved in the production of F pili and *tra*T is involved in surface exclusion. Also, if a pilot protein were to be cleaved, as in the case of the A protein of R17 (Krahn *et al*. 1972), the pilot protein may be

Table 2 Molecular Weight of the *tra* proteins

| Cistron | Molecular weight of protein |
|---|---|
| *tra*M | 13,000 |
| *tra*J | 23,500 |
| *tra*A | 13,700 |
| *tra*L | 11,000 |
| *tra*E | 19,000 |
| *tra*K | - |
| *tra*B | 29,000 |
| *tra*C | 78,000 |
| *tra*F | 25,000 |
| *tra*H | 40,000 |
| *tra*S | 18,000 |
| *tra*T | 25,000 |
| *tra*D | 77,000 |
| *tra*I | - |

about 67,000 to 68,000 in molecular weight, if two of the protein spots on the two dimensional autoradiograms are cleavage products of one protein. Two of the _tra_ products, the _tra_C protein, which is involved in F pilus synthesis and the _tra_D protein, which has been postulated (Paranchych, 1975) to be required for penetration of RNA and DNA through the cell membrane, have molecular weights about 77,000 so these are possible candidates for a pilot protein which might be cleaved. Since the _tra_C protein is involved in F pilus synthesis, it is unlikely to be this protein, but the _tra_D protein may be a good prospect.

In the retraction model of Curtiss (1969) and Marvin and Hohn (1969), the donor and recipient cells come together so that their membranes fuse during conjugation. Achtman (Achtman _et_ _al_. 1978b) has also suggested that wall to wall contact may occur before DNA transfer takes place, although he does not state that membrane fusion is a necessary component. When the cells separate after the completion of DNA transfer, an exchange of protein could occur between the donor and recipient cell membrane. It is possible, then, that transfer of membrane proteins could occur during conjugation. The major proteins of the outer membrane of _E_. _coli_ are the matrix protein, pOmpA, and lipoprotein (DiRienzo _et_ _al_. 1978). The molecular weights of these proteins plus the minor proteins of the outer membrane, most of which function in uptake of metabolites and/or as receptors for various colicins and bacteriophages, are given in Table 3. pOmpA is a heat modifiable protein, the molecular weight of which changes irreversibly from 29,500 to 34,700 upon heating to temperatures greater than 50° (Reithmeier and Bragg, 1977). The molecular weights of both the native and heat

Table 3 Molecular Weights of Outer Membrane Proteins of *E. coli*

| Protein | Molecular Weight |
|---|---|
| Matrix protein | 36,500 |
| pOmpA | 28,000 |
| lipoprotein | 7,000 |
| 83K | 83,000 |
| feuB | 81,000 |
| cit | 80,000 |
| tonA | 78,000 |
| cir | 74,000 |
| bfe | 60,000 |
| lamB | 55,000 |
| tsx | 27,000 |
| Protein G | 15,000 |
| Protein D | 80,000 |
| Phospholipase A1 | 29,000 |

modifiable forms of pOmpA have not yet been agreed upon, and so the values cited vary among authors. The matrix protein, of molecular weight 36,500, has an isoelectric point of 5.9 when determined in the presence of urea (Rosenbusch, 1974).

A comparison of the molecular weights of the spots that were visualized on the two dimensional gels with the molecular weights of the outer membrane proteins reveals a possible matching of spots with outer membrane proteins. Spot 1 from the two dimensional gels, having a molecular weight of 33,400 and a pI of 5.8 could match with the matrix protein (pI 5.9 and MW 36,500). This was the most intense spot seen in the autoradiograms and since the matrix protein is the most abundant of the outer membrane proteins, this may be a valid comparison. Either Spot 2 or Spot 3 from the two dimensional gels could be pOmpA from the outer membrane, on the basis of molecular weight. The protein samples were heated at 70° before applying to the IEF gel of the first dimension, thus, pOmpA would be converted to the higher molecular weight form. A more definite assignment must await the determination of the isoelectric point of pOmpA. On the basis of the foregoing, therefore, the proteins detected on the autoradiogram could be outer membrane proteins, resulting from some form of limited membrane fusion during conjugation.

This study has shown that protein transfer does occur during conjugation, with at least three proteins being transferred from the donor to the recipient cell. These findings argue against Brinton's condution and conveyor belt models of F pilus function as possible models, since these do not involve any protein transfer. On the other hand, both the retraction and carrier models predict the transfer of proteins. With regard to the latter model, the observed results could be explained,

in part, by postulating that the traD gene product acts as a pilot protein which is cleaved into two smaller polypeptides during transfer. Alternatively, it is also possible to suggest that the transferred proteins are derived from the outer membrane of the donor cell. The one spot from the two dimensional gels, with a pI of 5.8 and molecular weight of 33,400 matches very closely with the matrix protein of the outer membrane of *E. coli* which has a pI of 5.9 and molecular weight of 36,500. Although this assignment cannot be definitely made until the protein detected on the two dimensional gels is further characterized, if this spot is the matrix protein, it would indicate that membrane fusion must occur and would provide support for some form of retraction model. However, the cells may come into wall to wall contact by a method other than pilus retraction, so the presence of the matrix protein in the recipient cell would neither prove nor disprove the retraction model, but would indicate that membrane fusion must occur by some means during conjugation.

This study has shown that protein transfer from donor to recipient cells does occur during conjugation. These findings are consistent with two models for bacterial conjugation, namely, the retraction model of Curtiss (1969) and Marvin and Hohn (1969) and the carrier model suggested by Paranchych. Further experiments must be performed to distinguish between these two possibilities.

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